

**Bond University**

## **DOCTORAL THESIS**

**Intravesical gemcitabine treatment: repercussions on normal bladder function.**

Farr, Stefanie

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PhD Thesis

by

Stefanie Elizabeth Farr

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**INTRAVESICAL GEMCITABINE TREATMENT:  
REPERCUSSIONS ON NORMAL BLADDER FUNCTION**

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Submitted in total fulfilment of the requirements of the degree of  
Doctor of Philosophy by Research

July 2015



## ABSTRACT

Intravesical treatment for non-muscle invasive bladder cancer involves the direct instillation of immunotherapy or chemotherapy into the bladder. While this approach limits systemic absorption, patients undergoing this localised treatment frequently report significant urological side effects, including increased frequency and urgency of urination, haematuria and dysuria. A relatively new drug used for bladder cancer is gemcitabine, which has shown an improved efficacy and toxicity profile with comparison to the first-line chemotherapy mitomycin C in patients. Elucidating the effects of gemcitabine on the normal cells and changes in the normal function of the bladder may reveal possible targets for preventing, alleviating or treating the adverse urological effects associated with this treatment.

The cytotoxic effect of gemcitabine alone and in combination with hyperthermia treatment was examined on cultured non-malignant and malignant human urothelial cells, and compared to mitomycin C. Luminal gemcitabine treatment on full thickness porcine bladder sections examined the immediate histological and functional effects on the urothelium and detrusor muscle. Finally, single and repeated intravesical instillations of gemcitabine in mice examined the changes in voiding behaviours and *ex vivo* bladder function. Chemical, mechanical and electrical stimuli were used to investigate and compare the responses of control and treated tissues.

The potency of gemcitabine on malignant urothelial cells was >10,000-fold greater than its potency on non-malignant cells. This effect is attributed in part to the enhanced reactive oxygen species production induced by gemcitabine, and the enhanced presence of the human equilibrative nucleoside transporters in malignant cells with comparison to non-malignant urothelial cells. Gemcitabine also induced increased release of inflammatory cytokines from cultured urothelial cells, and these effects were not potentiated by hyperthermia. Luminal gemcitabine sloughed urothelial cells from porcine tissue, resulting in decreased ATP but enhanced prostaglandin E<sub>2</sub> release from the urothelium. Repeated intravesical gemcitabine treatment with subsequent recovery periods in mice increased voiding frequency, enhanced urothelial ATP and prostaglandin E<sub>2</sub> release but depressed detrusor contractile responses mediated by efferent nerve stimulation. Taken together, these results suggest that intravesical gemcitabine induces a painful and overactive bladder phenotype in patients through a combination of enhanced urothelial and inflammatory mediators and altered efferent nerve activity, which may sensitise afferent nerves and reduce detrusor muscle contraction respectively.



## **DECLARATION**

This thesis is submitted to Bond University in fulfilment of the requirements of the degree of Doctor of Philosophy by Research.

I declare that the research presented within this thesis is a product of my own original ideas and work, and contains no material which has previously been submitted for a degree at this university or any other institution, except where due acknowledgement has been made.

Stefanie Elizabeth Farr

3<sup>rd</sup> July 2015



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## PUBLICATIONS

Abstracts as a result of this thesis:

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Farr, S., McDermott, C. Chess-Williams, R. (2014) Gemcitabine enhances release of ATP but not acetylcholine or PGE<sub>2</sub> from bladder urothelial cells. Clinical Oncology Society of Australia, Melbourne 2014. Published in Asia-Pacific Journal of Clinical Oncology, Special Issue: COSA's 41st Annual Scientific Meeting v.10 S8 pp134 (No. 215). Available online at: <http://cosa-2014.m.asnevents.com.au/schedule/session/4459/abstract/18307>

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Stefanie E Farr, Catherine McDermott, Russ Chess-Williams (2012) Depressed contractile responses of the bladder detrusor and urothelium/lamina propria following luminal administration of the cytotoxic agent gemcitabine. Proc. ASCEPT Annual Meeting, Sydney 2012. (Abstract No. 622) Available online <http://www.asceptasm.com/wp-content/uploads/2013/07/ASCEPT-APSA-Poster-Abstracts-570-644.pdf>

Stefanie Farr, Catherine McDermott, Russ Chess-Williams. (2012) Administration of luminal gemcitabine and combined chemohyperthermia treatment depress contractile and relaxant responses of the bladder tissue. 4<sup>th</sup> National Symposium on Advances in Gastrointestinal & Urogenital Research, Sydney 2012

# TABLE OF CONTENTS

<b>Abstract</b>	<b>..... i</b>
<b>Declaration</b>	<b>..... iii</b>
<b>Acknowledgements</b>	<b>..... v</b>
<b>Publications</b>	<b>..... vii</b>
<b>Table of Contents</b>	<b>..... viii</b>
<b>Abbreviations</b>	<b>..... xi</b>
<b>Chapter 1: General Introduction</b>	<b>..... 1</b>
1.1 The Function of the Urinary Tract	..... 3
1.2 Bladder Cancer	..... 30
1.3 Gemcitabine	..... 40
1.4 Summary	..... 49
1.5 Research Questions and Hypothesis	..... 50
<b>Chapter 2: General Methods</b>	<b>..... 53</b>
2.1 Assays for Urothelial Mediator Release	..... 55
2.2 Chemicals and Drugs	..... 58
2.3 Histology	..... 60
2.4 Analysis	..... 62
<b>Chapter 3: Effects of Gemcitabine on Cultured Human Urothelial Cells</b>	<b>..... 65</b>
3.1 Introduction	..... 67
3.2 Aims	..... 75
3.3 Materials and Methods	..... 76
3.4 Results	..... 88
3.5 Discussion	..... 112

<b>Chapter 4: Effects of Gemcitabine on the Function of Isolated Porcine Bladder</b>	<b>..... 124</b>
4.1 Introduction	..... 126
4.2 Aims	..... 131
4.3 Materials and Methods	..... 132
4.4 Results	..... 136
4.5 Discussion	..... 161
 <b>Chapter 5: Effects of Gemcitabine In Vivo and on Isolated Whole Bladder Function in Mice</b>	 <b>..... 177</b>
5.1 Introduction	..... 179
5.2 Aims	..... 185
5.3 Materials and Methods	..... 186
5.4 Results	..... 197
5.5 Discussion	..... 218
 <b>Chapter 6: General Discussion</b>	 <b>..... 229</b>
 <b>References</b>	 <b>..... 249</b>



## ABBREVIATIONS

$\alpha,\beta$ -mATP: alpha, beta methylene ATP  
ACh: acetylcholine  
AChE: acetylcholinesterase  
ADP: adenosine 5'-diphosphate  
AP-1: activator protein-1  
AR: adrenoceptor  
ATP: adenosine 5'-triphosphate  
 $\text{Ca}^{2+}$ : calcium  
cAMP: 3'-5'-cyclic adenosine monophosphate  
CGRP: calcitonin gene-related peptide  
COX: cyclooxygenase  
DAG: diacylglycerol  
dCK: deoxycytidine kinase  
DFV: discoidal fusiform vesicle  
DRG: dorsal root ganglia  
EFS: electrical field stimulation  
EGF: epidermal growth factor  
EGFR: epidermal growth factor receptor  
GAG: glycosaminoglycans  
GSH: glutathione  
 $\text{H}_2\text{O}_2$ : hydrogen peroxide  
IL: interleukin  
 $\text{IP}_3$ : inositol trisphosphate  
 $\text{K}^+$ : potassium  
KCl: potassium chloride  
L-NNA: L- $\text{N}^G$ -Nitroarginine  
MIBC: muscle invasive bladder cancer  
NA: noradrenaline  
 $\text{Na}^+$ : sodium  
NANC: non-adrenergic, non-cholinergic  
NGF: nerve growth factor  
NMBPR: S-(4-nitrobenzyl)-6-thioinosine  
NMIBC: non-muscle invasive bladder cancer  
NO: nitric oxide  
NOS: nitric oxide synthase  
 $\text{PGE}_2$ : prostaglandin E2  
PLC: phospholipase C  
PMC: pontine micturition centre  
ROS: reactive oxygen species  
RT-PCR: reverse transcription polymerase chain reaction  
SEM: standard error of the mean  
TCC: transitional cell carcinoma  
TK2: thymidine kinase 2  
TNF: tumor necrosis factor  
TRP: transient receptor potential  
TTX: tetrodotoxin  
TUR: transurethral resection  
UDIF: urothelium derived inhibitory factor  
UP: uroplakin



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## **CHAPTER 1:**

### **GENERAL INTRODUCTION**

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## 1.1 THE FUNCTION OF THE URINARY TRACT

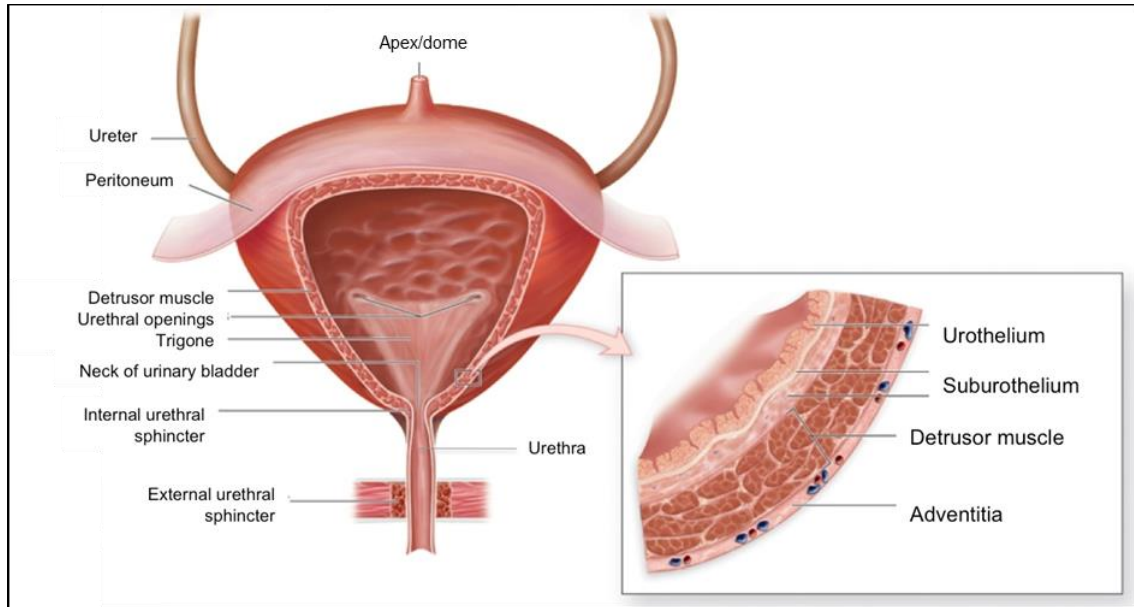
The urinary tract is the organ system that produces, stores and expels urine from the body. The kidneys and ureters comprise the upper urinary tract, while the lower urinary tract describes the bladder and urethra, and includes the prostate in males (Patel and Chapple, 2008). The kidneys function to filter the blood of toxins, metabolites and wastes, maintaining the homeostasis of fluid within the body. The wastes are transported by the ureters to the bladder, where it is stored until it can be expelled through the urethra. This process is called micturition, co-ordinated through the pelvic, hypogastric and pudendal nerves (de Groat and Yoshimura, 2001).

### GROSS ANATOMY OF THE BLADDER

The urinary bladder is a hollow, spherical muscular organ that acts as a temporary reservoir for urine. The bladder, along with the urethra and urethral sphincters make up the lower urinary tract, and is positioned retroperitoneally on the pelvic floor posterior to the pubic bone (Marieb and Hoehn, 2007). In males, the prostate surrounds the bladder neck where it empties to the urethra, while in females the bladder sits anterior to the vagina and uterus. There are specific regions of the bladder as seen in **Figure 1.1**, the apex, often termed the dome, a superior surface and two inferolateral walls, all covered in folded epithelia called rugae (Seeley et al., 2007). The triangular area at the base of the bladder encompassed by the openings of the ureters and the urethra is called the trigone (Standring, 2008). At the apex of the trigone (also known as the bladder neck) lies the internal urethral sphincter, a band of smooth muscle under involuntary control, however, no well-defined internal urethral sphincter has been found in females (Colleselli et al., 1998, Seeley et al., 2007).

There are three layers of the bladder wall (**Figure 1.1**): the outer adventitial layer, the muscular layer and an inner epithelial layer that is continuous with the ureters above and the urethra below. The inner epithelial layer, termed the urothelium, primarily acts as a barrier to water and solutes between the lumen and underlying muscle. Recent evidence supports the role of the urothelium in detecting and responding to mechanical, chemical and thermal stimuli, which will be discussed in detail further in this report (Birdier, 2006). The urothelium can communicate with

underlying cells, including those found in the suburothelium and muscle layer, through the presence of receptors and secretion of mediators. The suburothelium is a collagen matrix containing blood vessels, myofibroblasts and afferent nerves.

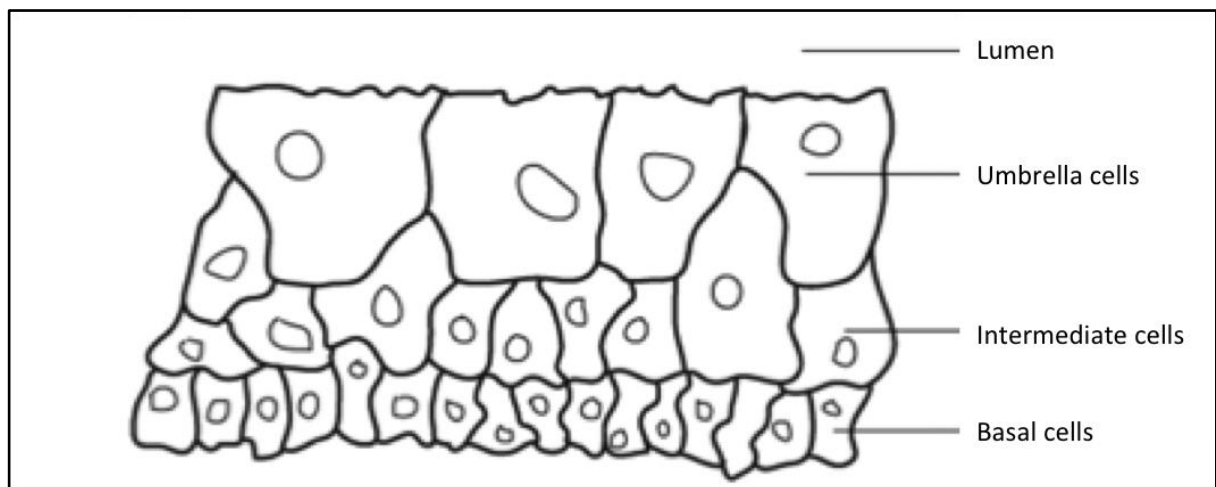


**Figure 1.1: Gross anatomy of the urinary bladder (Mckinley, 2008)**

Below the suburothelium lies the bladder muscle, made up of an inner layer of longitudinal fibres, a middle layer of circular fibres and an outer layer in which the fibres run longitudinally and parallel to the inner layer (Stevens and Lowe, 2005). The muscle layers are collectively described as the detrusor and function in the contraction of the bladder during micturition. The outer adventitial layer is a thick fibrous membrane that covers the bladder with the exception of the superior surface, where the peritoneum lies.

## THE UROTHELIUM

The urothelium is an epithelial lining that separates the suburothelium and bladder muscle from the contents of the bladder lumen. It is continuous with the distal portion of the urinary tract, including the ureters, upper urethra and glandular ducts of the prostate (Apodaca, 2004). Due to cyclical changes in volume and pressure of the bladder during the filling and micturition phases, the main role of the urothelium is to form a distensible barrier that regulates exchange of ions, solutes and water. However, the close proximity of the bladder nerves and bladder myofibroblasts, as well as the presence of receptors and ion channels on the urothelial cells similar to those found on afferent nerves, indicate that the urothelium plays an active role in sensory detection and communication, which will be discussed later in this report (Birder et al., 2010a). There are three cell types in the urothelium, the umbrella, intermediate and basal cell layers, as seen in **Figure 1.2**. These cells are morphologically distinct, and play differing roles within the urothelium.



**Figure 1.2:** Schematic illustration of unstretched urothelium composed of three cell layers: umbrella, intermediate and basal cells (Birder, 2005)

### UMBRELLA CELLS

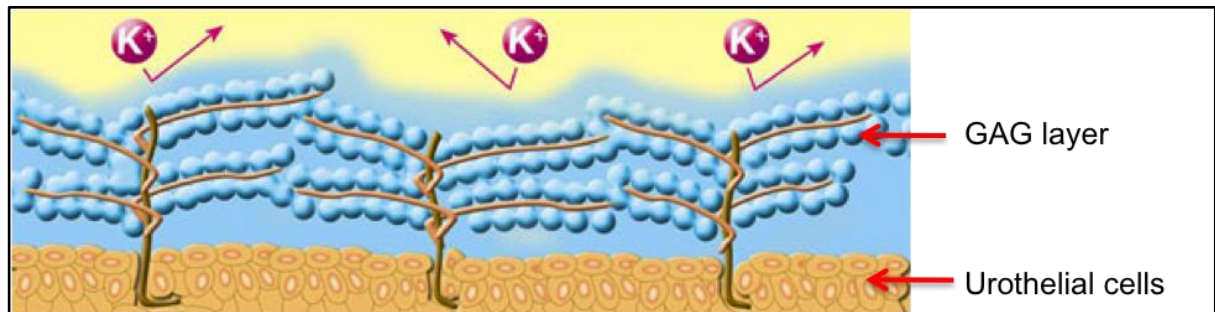
The inner most cell layer to the lumen of the bladder is the umbrella cell layer. They cast projections that reach the basement membrane of the urothelium, and are the largest cells, spanning 25-250 $\mu$ m in diameter (Apodaca, 2004). In the human, umbrella cells are mono- and multinucleate, and their morphology is reliant on the filling state of the bladder (Morrow et al.,

2008). In the relaxed state, umbrella cells are cuboidal in shape, and become stretched and squamous during filling.

The barrier function of the urothelium is dependent upon the presence of the apical membrane. This impermeable layer distinctly defines each cell, and is scalloped in appearance due to the presence of hinge areas and intervening transmembrane plaques (Apodaca, 2004). The membrane associated with the plaque regions is detergent insoluble, likely due to the rich lipid composition reported to be similar to myelin (Hicks et al., 1974). The arrangement of the membrane plaques is such that the outer leaflet is twice as thick as the inner, termed the asymmetric unit membrane (AUM) (Apodaca, 2004). The transmembrane proteins that make up the AUM are the family of uroplakins, which are also found in the cytoplasm of bladder umbrella cells within discoidal or fusiform vesicles (DFV) (Yu et al., 1990). Four types of uroplakins (UP) are arranged to form a heterotetramer, including UP Ia/UP II and UP Ib/ UP III (Lee, 2011), covering almost all of the mature DFV. These vesicles undergo exocytosis in response to stretch of the bladder during filling by fusing to the cell membrane to deliver uroplakins and other essential proteins (Truschel et al., 2002), which allow the umbrella cells to distend, preventing the AUM from rupturing (Lee, 2011). Uroplakins have been found to play a role in water and solutes transport, as studies into uroplakin knock-out mice have found increased permeability in the bladder to water and urea (Hu et al., 2002). It is the release of ATP with stretch that initiates a signal cascade which begins the exocytosis process (Kreft et al., 2009a). The role of ATP as a signalling messenger in the bladder will be discussed in detail later, along with other urothelial mediators.

The presence of the AUM, uroplakins and cytokeratins on the apical cells of the urothelium indicative of urothelial differentiation (Veranic et al., 2004). While the normal turnover rate for urothelial cells is up to 40 weeks (Jost, 1989), injury to the bladder such as urinary infections induce an immediate regeneration process. Production of new umbrella cells occurs through the maturation of the underlying intermediate cells, which increase their expression of uroplakins and develop tight junctions between stratum (Lavelle et al., 2002). The presence of multi-nucleate umbrella cells has been proposed to be due to the fusing of two or more intermediate cells (Peter, 1978). The presence of surface glycosaminoglycans (GAGs) on the apical side of

the umbrella cells creates the principle barrier to permeability by modulating molecule and ion movement (Parsons et al., 1990, Lilly and Parsons, 1990). GAGs are strands of negatively charged polysaccharides, which attach to a core protein to form proteoglycans and attract cations and water molecules to form a mucus-like permeability barrier, as shown in **Figure 1.3**.



**Figure 1.3: Urothelium with GAG layer, preventing irritants from contact with the bladder wall**

GAGs are also thought to act as an anti-adherence factor, forming a defence barrier against bacteria, proteins and other small molecules (Parsons et al., 1979, Birder, 2011). Disruption of this GAG layer and the consequent loss/damage to the mucus barrier has been associated with infection, interstitial cystitis, radiation and chemical cystitis and cancer of the bladder (Parsons, 2007).

Umbrella cells are interconnected with gap junctions, and project transmembrane proteins that adhere to the basement membrane of the urothelium. Using atomic force microscopy, Kreplak and colleagues (2007) found that the membranes of aligning cells are fused to form a zipper, contributing to the barrier function of the urothelium. Desmosomes, complexes of cell adhesion proteins, connect the layer of intermediate cells to one another and to the overlying umbrella cells. Gap junctions within the layer of intermediate cells have been found to be distributed randomly, similarly to the basal cell layer of the urothelium (Peter, 1978).

## **INTERMEDIATE AND BASAL CELLS**

The single-nuclei, pyriform shaped intermediate cells make up a transiently thick layer comprised of between one to several stratum, dependent on the state of the bladder (Jost et al., 1989). It is thought that adjacent intermediate cells slide past each other during filling, however

whether this is due to reversible breakage of cell connections is unknown (Khandelwal et al., 2009). The intermediate cells directly deep to the umbrella cell layer are partially differentiated and can express uroplakins and vesicles, similar to the superior layer (Wu et al., 2009, Apodaca, 2004). The basal cells of the urothelium also have the ability to differentiate further to form intermediate cells (Mysorekar et al., 2002), as a result of infection or disruption to the superior urothelial layers. The single basal cell layer of the urothelium sits atop the basement membrane and beneath the intermediate cell layer, attached by hemidesmosomes and desmosomes respectively (Woldemeskel et al., 1998).

## **SUBUROTHELIUM/LAMINA PROPRIA**

Beneath the urothelium lies the suburothelium, often termed the lamina propria. The suburothelium is a collagen matrix that contains afferent and efferent nerve terminals, blood vessels of the bladder, fibroblasts and a group of specialised cells, the interstitial cells (Fry et al., 2007, Sui et al., 2002).

## **INTERSTITIAL CELLS/MYOFIBROBLASTS**

Interstitial cells, also known as myofibroblasts, have structural characteristics of both smooth muscle cells and fibroblasts and resemble those found in the gut, where they are called interstitial cells of Cajal (McCloskey, 2010). Interstitial cells were first discovered within the bladder in 1996 (Smet et al.), and their presence in close proximity to nerve endings has since been confirmed through c-Kit antibody marking and vimentin immunolabelling (Davidson and McCloskey, 2005). Suburothelial interstitial cells are stellate shaped cells, connected by gap junctions (Fry et al., 2007). While these cells are also found within the detrusor layer of the bladder, they are comparatively different in distribution and morphology, the detrusor interstitial cells being elongated cells arranged in circular and longitudinal orientation (McCloskey, 2010, Wiseman et al., 2003).

Interstitial cells in other smooth muscle tissues such as the gastrointestinal tract act as pacemaker cells (Sui et al., 2002, Sanders, 1996), and generate phasic myogenic contractions without neural stimulation, however whether this is the case with bladder interstitial cells has not yet been elucidated (Thornbury et al., 2011, Birder et al., 2010a). Interstitial cells form a network through connexin 43-containing gap junctions, establishing cell-to-cell communication enabling these cells to act as a functional syncytium (Sui et al., 2002). It has been proposed that these cells in the bladder act as mediators of signal transduction between the urothelium and nerves, due their ability to respond to various neurotransmitters such as ATP, acetylcholine, and nitric oxide (NO) (Birder et al., 2010b, Sanders, 1996, Wiseman et al., 2003). The ion channels and receptors present on interstitial cells of the bladder include calcium, sodium and potassium channels; transient receptor potential cation channel V1 (TRPV1), purinergic and cholinergic receptors (Grol et al., 2009, McCloskey, 2006, Fry et al., 2007, Hanna-Mitchell and Birder, 2008). These receptors will be discussed in detail later on in this report.

## **INNERVATION OF THE BLADDER**

The phases of storage and micturition of the bladder are mediated by a complex pattern of afferent and efferent signalling through the pelvic, hypogastric and pudendal nerves. During storage, afferent axons transmit signals regarding the volume and stretch of the bladder. The micturition reflex is stimulated by increased afferent firing to the sacral region of the spinal cord, which in turn stimulates efferent activity, mediated voluntarily by the cerebral cortex (de Groat and Yoshimura, 2001). Signals travelling through the sympathetic, parasympathetic and somatic neurons coordinate the contraction of the bladder and relaxation of the bladder outlet (the bladder neck, urethra, internal and external sphincters).



## AFFERENT NERVES

The afferent nerves carry sensory information about the fullness of the bladder and bladder outlet through the pelvic, pudendal and hypogastric nerves through the dorsal root ganglia (DRG) to the upper lumbar and sacral regions of the spinal cord, as shown in **Figure 1.4** (Fowler et al., 2008, Jänig and Morrison, 1986). The main afferents involved in the micturition process are the small myelinated A- $\delta$  and unmyelinated C fibres, which are carried through the pelvic (majority) and hypogastric nerves, terminating in the bladder wall (Andersson et al., 1999, Ford et al., 2006).

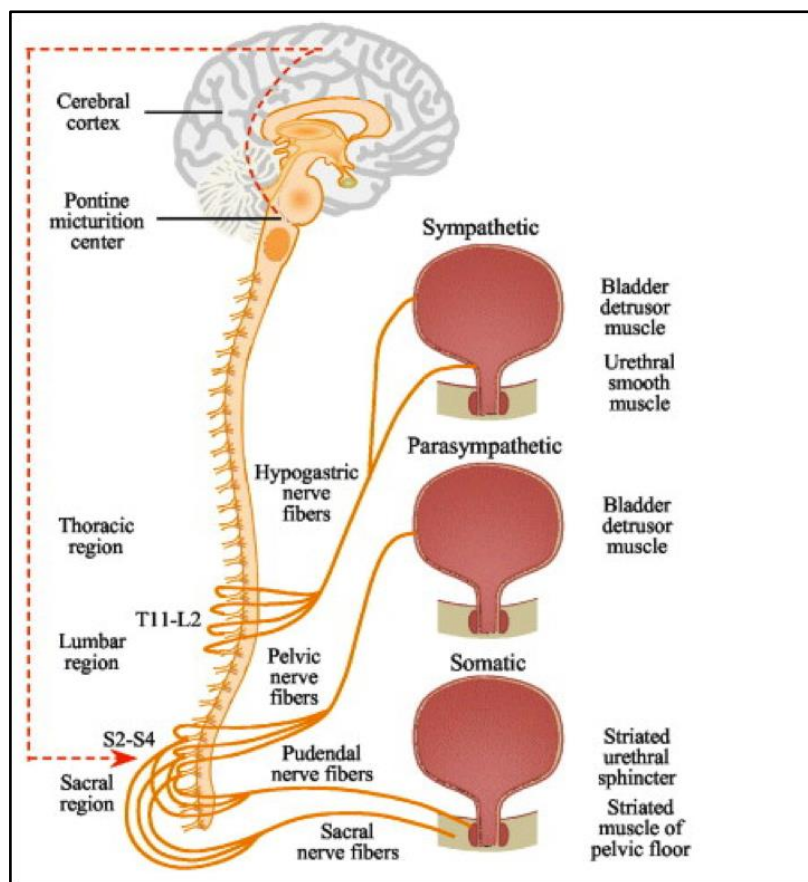


Figure 1.4: Innervation of the bladder and bladder outlet. The hypogastric (sympathetic), pelvic (parasympathetic) and pudendal (somatic) nerves coordinate the micturition process of the bladder and urethra. Primary cell bodies of A- $\delta$  and C-fiber afferents of the pelvic and pudendal nerves are contained in lower lumbar and sacral dorsal root ganglia (DRG), and afferent innervations in the hypogastric nerve arises in rostral lumbar DRG (reprinted with permission Kanai and Andersson, 2010)

These mechanosensitive afferents originating in the bladder wall convey information regarding the degree of stretch of the bladder, travelling through the pelvic nerve to the second order neurons in the spinal cord. The A- $\delta$  fibres have been found to instantly respond to very small changes in pressure through passive distension and active contraction of the bladder wall (Fowler et al., 2008, Wyndaele, 2010). Additionally, these afferents have chemosensitive properties, exhibiting a more sensitive response with exposure to irritants. In comparison, the slower unmyelinated C fibres that originate in the urothelium and underlying suburothelium have very high thresholds and thus have little contribution during normal bladder filling (de Groat, 2006, Häbler et al., 1990, Abrams et al., 2001). C fibres are sensitive to neurotoxins, capsaicin and resiniferatoxin, as well as tachykinins, NO, ATP, prostaglandins, endothelins and neurotrophic factors released in the bladder, shown in **Figure 1.5**, and are particularly active during periods of hypersensitivity due to inflammation or damage to the LUT (Abrams et al., 2001, Häbler et al., 1990, Maggi, 1990, de Groat, 2006).

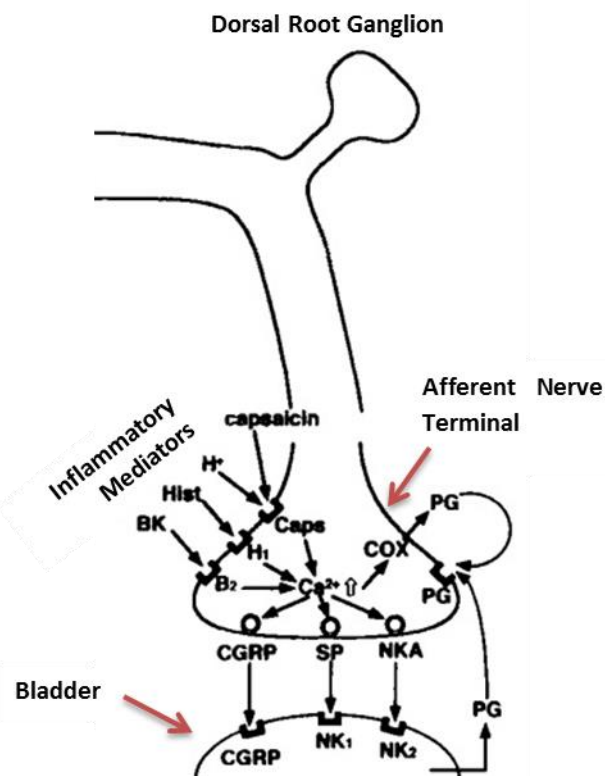


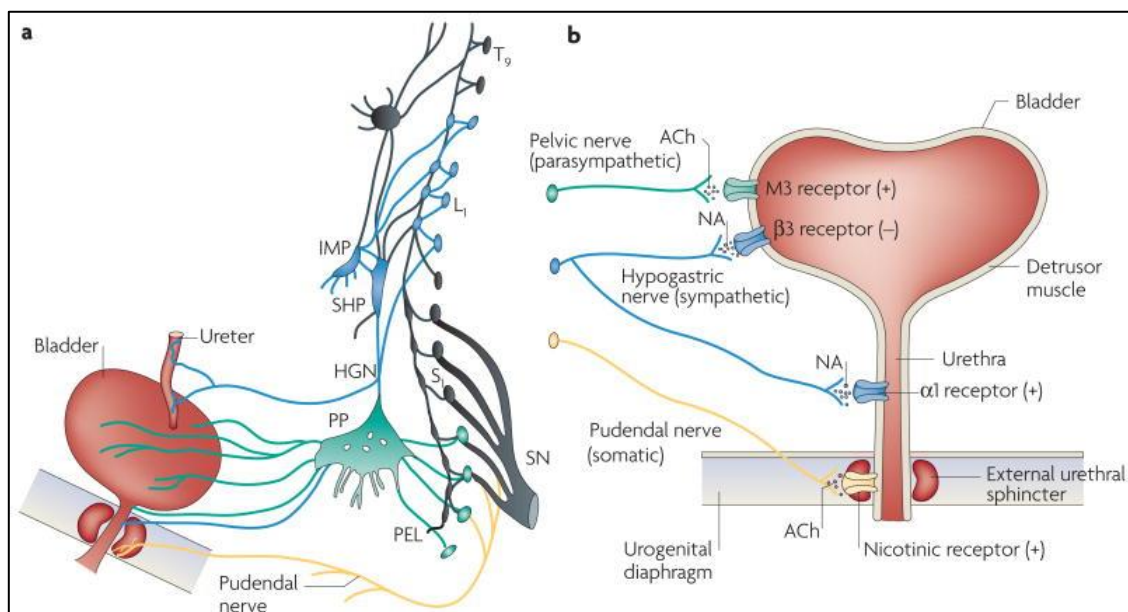
Figure 1.5: Interactions of neurotransmitters and chemical factors on bladder afferent pathways (especially C-fibre afferent nerves) originating in the bladder. During periods of bladder inflammation, bradykinins (BK), histamine (Hist) and protons act on their respective bradykinin receptor (B<sub>2</sub>), histamine receptor (H<sub>1</sub>) and vanilloid receptor (Caps) and induce an influx of calcium ions (Ca<sup>2+</sup>), triggering the production of neuropeptides including calcitonin gene-related peptide (CGRP), substance P (SP) and neurokinin A (NKA), which act on their respective CGRP and neurokinin 1 (NK<sub>1</sub>) and 2 (NK<sub>2</sub>) receptors on the bladder wall. Inflammation also initiates the production of prostaglandins (PG) by cyclooxygenase, which act in an autocrine manner on prostaglandin receptors to sensitize the afferent nerve terminals and stimulate the release of neurotransmitters (reprinted with permission from Yoshimura and de Groat, 1997)

The sensory somatic nerves ending in the striated muscle of the external urethral sphincter travel through the pudendal nerve to the sacral region of the spinal cord, while the sensory information from the urethra is transmitted through the hypogastric nerve.

Nociceptors present in the bladder are unmyelinated afferent fibres that monitor mechanosensitivity during over distension of the bladder, and transmit signals of pain. These are activated by increased release of ATP, which will be discussed in detail later.

## EFFERENT NERVES

The efferent neural pathways are responsible for the voluntary control of micturition process, involving the somatic, parasympathetic and sympathetic nerve pathways. The somatic and parasympathetic innervations originate in the sacral part of the spinal cord, travelling through the pudendal and pelvic nerves respectively, while the sympathetic nerves to the bladder pass via the hypogastric nerve from the thoracolumbar segments, as seen in **Figure 1.6** (Fowler et al., 2008).



**Figure 1.6: Efferent neural pathways of the urinary bladder.** a) Blue sympathetic fibres originate in the thoracolumbar spinal segments T10-L2 and run through the inferior mesenteric plexus (IMP) and hypogastric nerve (HGN) to the base of the bladder and the urethral neck. The green fibres represent the parasympathetic nerves that arise between the S2-S4 segments, travelling through the pelvic nerve (PEL) via the pelvic plexus (PP) to the bladder wall. Somatic nerves (yellow fibres) arise from the sacral spinal segments S2-S4 and pass through the pudendal nerve to the external urethral sphincter. b) The post-ganglionic sympathetic nerve fibres release noradrenaline, which acts on the  $\beta$  and  $\alpha$  adrenoceptors on the bladder and urethra respectively. The parasympathetic and somatic postganglionic axons release acetylcholine, which respectively stimulate the muscarinic receptors in the detrusor muscle and nicotinic receptors on the external urethral sphincter (reprinted with permission from Fowler et al., 2008).

### *PARASYMPATHETIC SUPPLY*

The micturition reflex is initiated through afferent activity monitoring the stretch and fullness of the bladder, which is conveyed to the pontine storage centre via the brainstem and midbrain. With increasing afferent activity, the pontine micturition centre is excited, which in turn stimulates the parasympathetic pathway that leads to detrusor contraction (Drake et al., 2010). The parasympathetic bladder nerves originate in the spinal cord between the S2-S4 sacral segments, with the post-ganglionic nerves sending excitatory stimulation to the bladder detrusor muscle (Andersson et al., 1999, Birder et al., 2010a), as seen in **Figure 1.6**.

The majority of the postganglionic parasympathetic fibres release acetylcholine, acting on the muscarinic receptors of the bladder muscle, inducing contraction. In addition to the release of acetylcholine, parasympathetic stimulation causes the release of non-adrenergic, non-cholinergic (NANC) transmitters including ATP (Burnstock, 2009). The release of these transmitters leads to the activation of their respective receptors, initiating and maintaining detrusor contraction in the bladder, while relaxing the bladder outlet for the expulsion of urine (Sellers and Chess-Williams, 2012).

Besides the excitatory effect on the detrusor muscle, parasympathetic stimulation plays a role in the inhibition of the urethral smooth muscle. Previous studies found that urethral tissue relaxed in response to neural stimulation, abolished by the addition of tetrodotoxin (TTX), implying the release of these transmitters is dependent on nerve activity (Andersson et al., 1992, Persson and Andersson, 1992, Andersson et al., 1983). Nitric oxide (NO), released with parasympathetic stimulation to the proximal urethra, is responsible for the relaxation of the urethral smooth muscle, in particular the internal urethral sphincter (Bennett et al., 1995, de Groat and Yoshimura, 2001). These transmitters and receptors will be discussed in further detail later on in this report.

### *SYMPATHETIC SUPPLY*

The sympathetic innervation of the bladder begins with preganglionic axons originating from the thoracic spinal and extending to the upper lumbar segments (T10 - L2) (Kanai and Andersson,

2010), seen in **Figure 1.6**. These axons run through the inferior mesenteric plexus (ganglion) and hypogastric nerve to inhibit contraction of the detrusor wall allowing bladder filling, and the ganglia of the pelvic plexus via the pelvic nerve to induce contraction of the internal urethra (Lincoln and Burnstock, 1993).

The bladder base has a denser sympathetic innervation than the detrusor muscle (Michel and Vrydag, 2006). Sympathetic nerves release noradrenaline that act upon  $\beta$ -adrenoreceptors in the detrusor muscle, mediating relaxation and enhancing bladder compliance (Michel and Barendrecht, 2008). In the bladder outlet, noradrenaline acts upon  $\alpha$ -adrenoceptors, which increase muscle tone in the proximal urethra and the bladder base preventing any leakage of bladder contents (Ochodnický et al., 2013). In males, the presence of  $\alpha$ -adrenoceptors on the prostate enhances bladder outlet resistance, particularly in men with enlarged prostates (Michel and Vrydag, 2006). A further role of the sympathetic nerves of the bladder is to inhibit the parasympathetic pathway at spinal and ganglionic levels (Chapple et al., 2011).

Inhibition of detrusor tone by the sympathetic fibres of the bladder was first suggested by Gjone (1966) and Edvardsen (1968) after investigations into transected sympathetic nerves in cats led to increased spontaneous and reflex contractions. During filling, the parasympathetic ganglia of the bladder are suggested to be inhibited by the sympathetic stimulation of  $\alpha_2$ -adrenoceptors (de Groat, 2006, De Groat and Saum, 1972).

### *SOMATIC NERVES*

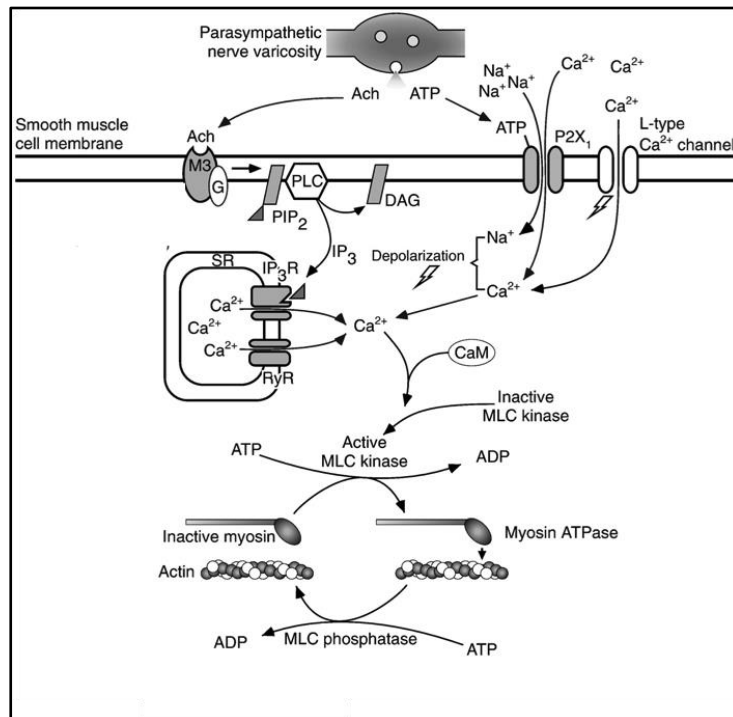
The somatic nerves of the bladder arise in Onuf's nucleus in the sacral segments S2-S4 of the spinal cord, and travel through the pudendal nerve to supply the striated muscle of the external urethral sphincter (Fowler et al., 2008), as seen in **Figure 1.6**. These cholinergic nerves voluntarily contract the external urethral sphincter muscle via acetylcholine acting upon nicotinic receptors (de Groat, 2006, Michel and Barendrecht, 2008).

## **DETRUSOR MUSCLE**

The role of the bladder to transiently store and excrete urine is mediated through complex interactions between the sensory and muscular components of the urinary tract. Contraction of the bladder muscle, the detrusor, is responsible for the rapid expulsion of urine. During filling, the detrusor cells relax and elongate over an extended period to accommodate the gradual increase in bladder contents. In comparison, the micturition reflex involves a synergistic contraction of the muscle cells over a relatively short period (Andersson and Arner, 2004).

The smooth muscle cells of the bladder are spindle shaped, single nucleated cells, organised into bundles of circular and longitudinal muscle called fascicles, surrounded by collagen-rich connective tissue (Andersson and Arner, 2004). John et al. (2003) discovered the presence of connexin 45 mRNA on human detrusor cells, suggesting the presence of electrically coupled gap junctions. They concluded that intercellular communications mediated through gap junctions might play a role in detrusor tone. Further investigations into connexin proteins in rodent bladders found a relationship between overactive detrusor and increased Cx-43 expression, indicating a link between intercellular communication and spontaneous contractions of the detrusor (Christ et al., 2003).

Like all other muscle types, the interaction of the contractile proteins actin and myosin are responsible for the tension development and shortening of the detrusor muscle (Gunst and Zhang, 2008). Compared to striated muscle, smooth muscle can maintain contractions more economically, using less ATP and contracting for longer periods (Mundy et al., 2000).



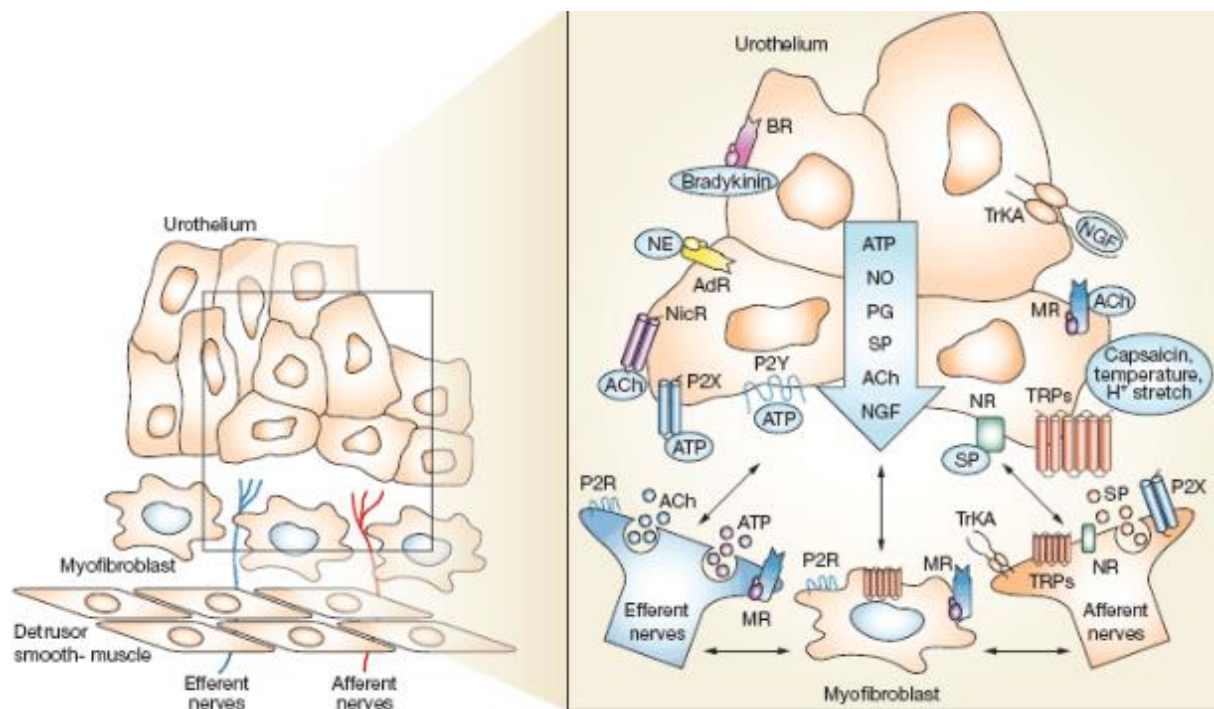
**Figure 1.7: The intracellular signalling pathways in detrusor muscle contraction (reprinted with permission from Fry et al., 2010).**

The release of neurotransmitters acetylcholine and ATP from parasympathetic nerves initiates a signalling cascade for the activation of actin-myosin cross bridge cycling in detrusor muscle, as shown in **Figure 1.7** (Fry et al., 2010). The G-coupled muscarinic M<sub>3</sub> receptor binds acetylcholine released from the parasympathetic nerves, initiating the formation of inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) from membrane phospholipids (PIP<sub>2</sub>) by the action of phospholipase-C (PLC). IP<sub>3</sub> initiates Ca<sup>2+</sup> release from intracellular Ca<sup>2+</sup> stores (sarcoplasmic reticulum, SR). Ca<sup>2+</sup> binds to calmodulin (CaM) to activate myosin light chain (MLC) kinase and phosphorylate and activate myosin to bind to actin. The binding of ATP to the purinergic P2X<sub>1</sub> receptor opens the ion and the L-type Ca<sup>2+</sup> channel, causing influx of Ca<sup>2+</sup> and Na<sup>+</sup> ions (Fry et al., 2010). Intracellular calcium from the sarcoplasmic reticulum and extracellular calcium bind to calmodulin, which in turn activates myosin, through the activation of the myosin light chain (MLC) kinase (See **Figure 1.7**). Cross bridge cycling between actin and myosin molecules occurs as long as myosin is activated and ATP is available for consumption.



## NEUROTRANSMITTERS, UROTHELIAL MEDIATORS AND RECEPTORS

The urothelium was previously thought to act solely as a barrier to water and solutes, protecting the underlying tissues of the bladder. The discovery by Hypolite and colleagues (1993) that the urothelium has a higher metabolic rate than the underlying detrusor muscle suggested a greater role for urothelial cells in bladder function than a barrier. The presence of muscarinic, purinergic and adrenergic receptors and the secretion of mediators including ATP, acetylcholine, prostaglandins and nitric oxide from urothelial cells indicate a role in coordinating responses to mechanical and chemical stimuli between the urothelium and underlying nerves, interstitial cells and detrusor muscle, as seen in **Figure 1.8** (Birder, 2011).



**Figure 1.8: Release of mediators from the urothelium and the interactions with underlying nerves, interstitial cells (myofibroblasts) and detrusor muscle.** Urothelial cells are stimulated by receptors or channels in a number of ways, including mechanical and osmotic stretch and via autocrine and paracrine neurotransmitter release. These neurotransmitters include adenosine triphosphate (ATP), nitric oxide (NO), prostaglandins (PG), substance P (SP), acetylcholine (ACh) and nerve growth factor (NGF). Receptors for these mediators include purinergic receptors (P2X, P2Y and P2R [purinergic 2 receptor unidentified subtype]), muscarinic receptors (MR), nicotinic receptors (NicR), receptor tyrosine kinase A (Trk-A) and neurokinin receptors (NR). Other receptors and channels present on the urothelium include the bradykinin receptor (BR) which binds bradykinin, adrenergic receptors (AdR) for [noradrenaline] norepinephrine (NE) and transient receptor potential channel (TRPs) which is sensitive to capsaicin, temperature, H<sup>+</sup> ions and stretch (reprinted with permission from Birder and de Groat, 2007).

## ACETYLCHOLINE AND CHOLINERGIC RECEPTORS

Increased parasympathetic activity during bladder emptying causes acetylcholine release, which in turn works in a paracrine and autocrine manner, acting on the bladder nerves and muscle and the urothelium respectively (Hanna-Mitchell et al., 2007, Chess-Williams, 2002). Acetylcholine is synthesised from the reversible reaction between choline and acetyl coenzyme A forming acetylcholine and coenzyme A, catalysed by the enzyme choline acetyltransferase.

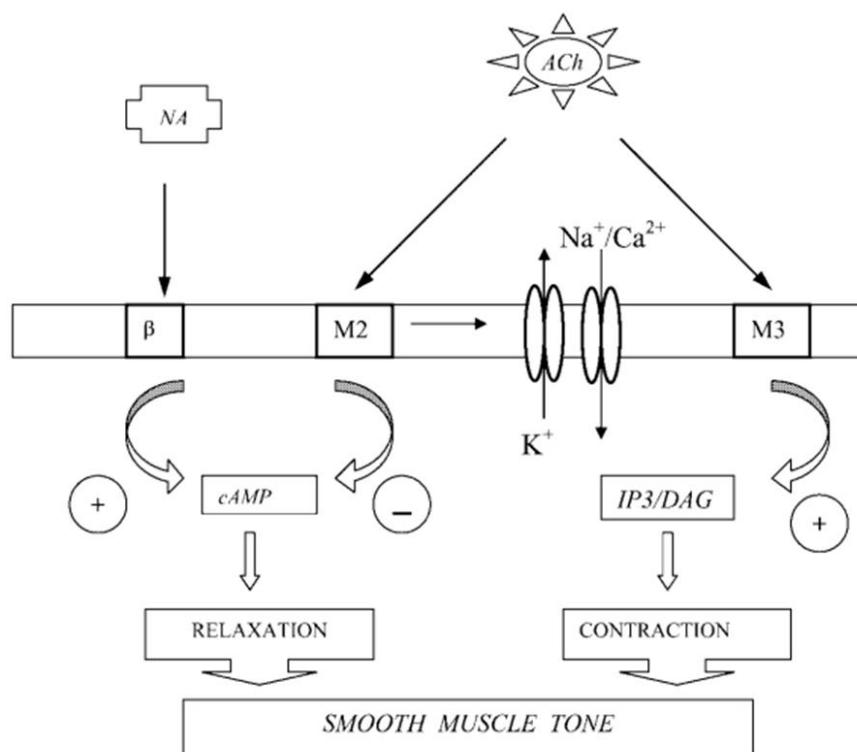
While acetylcholine released from storage vesicles in parasympathetic nerves stimulate the detrusor during voiding, studies have found that acetylcholine is also released during other phases of the bladder, from different sources (Apodaca, 2004). The release of acetylcholine from non-neuronal sources was confirmed when Yoshida and colleagues (2006) found that the basal release of acetylcholine was significantly decreased with the removal of the urothelial and suburothelial layer. Studies have since found urothelial cells possess the ability to synthesise, transport and metabolize acetylcholine in response to both chemical stimulation and mechanical stretch during bladder filling (Lips et al., 2007, Khandelwal et al., 2009, Hanna-Mitchell et al., 2007). Hanna-Mitchell et al (2007) first reported that urothelial cells from the rat bladder express the acetylcholine synthesizing enzymes choline acetyltransferase and carnitine acetyltransferase. As in neuronal cells, the synthesis of acetylcholine in urothelial cells requires uptake of choline from the extracellular compartment. However, it was found that urothelial cells lack the vesicular acetylcholine transporter, and the addition of brefeldin (a substance that blocks vesicle formation) had no effect on the hypotonic-induced release of  $^3\text{H}$ -acetylcholine from urothelial cells. Through the use of reverse transcription-PCR, the presence of rat electrogenic polyspecific organic cation transporter 3 was confirmed, indicative that acetylcholine is released from urothelial cells mediated via this mechanism, as in other epithelial cells including bronchial and placental (Hanna-Mitchell et al., 2007, Wessler and Kirkpatrick, 2001, Koepsell et al., 2003).

## MUSCARINIC RECEPTORS

Five types of muscarinic receptors have been pharmacologically defined, belonging to the family of G-protein coupled receptors classified as  $M_1 - M_5$  (Giglio and Tobin, 2009, Caulfield and Birdsall, 1998). Within the bladder, the receptors are expressed in three locations; the detrusor smooth muscle, the urothelium and on autonomic nerve endings (Tyagi et al., 2006).

The muscarinic receptors  $M_1$ ,  $M_3$  and  $M_5$  are coupled to the  $\alpha$  subunit of the Gq/11 proteins, which as previously described, stimulate phospholipase C (PLC) when activated, leading to an up-regulation of the second messengers inositol triphosphate ( $IP_3$ ) and diacylglycerol (DAG) leading to the influx of extra- and intracellular  $Ca^{2+}$  ions and consequent contraction (Caulfield and Birdsall, 1998). In comparison, the  $M_2$  and  $M_4$  receptors cause contraction indirectly, by inhibiting the relaxation by noradrenaline released from sympathetic nerves. These receptors are coupled via the  $G_i$  and  $G_o$   $\alpha$  subunits, and when stimulated, inhibit adenylate cyclase, reducing 3'-5'-cyclic adenosine monophosphate (cAMP). This process is shown schematically in

**Figure 1.9.**



**Figure 1.9:** Stimulation of the  $M_2$  and  $M_3$  receptors induce smooth muscle contraction by inhibition of cyclic AMP reversing relaxation and stimulation of second messengers  $IP_3$  and DAG (Uchiyama and Chess-Williams, 2004).

In the detrusor muscle the M<sub>2</sub> receptor is the most populous, however it is the M<sub>3</sub> receptor that mediates the contractile response in all species examined, including human (Chess-Williams, 2002). The M<sub>2</sub> receptor has been proposed to regulate the detrusor muscle during conditions such as M<sub>3</sub> dysfunction or desensitization (Braverman et al., 2006) or significant sympathetic activity, as well as mediating the acetylcholine induced release of prostaglandin E<sub>2</sub> in the urothelium (Nile and Gillespie, 2012). Additionally, ATP is co-released with acetylcholine in the bladder, and binding to P2X<sub>1</sub> receptors on the detrusor, it initiates a fast transient contraction through influx of calcium. In contrast, muscarinic receptor stimulation by acetylcholine released from the parasympathetic nerves induces prolonged contraction to maintain bladder emptying by opening inositol triphosphate channels (IP<sub>3</sub>) mediated channels, causing intracellular calcium release from the sarcoplasmic reticulum.

Muscarinic receptors in the bladder have been found on both the parasympathetic and sympathetic nerve endings, and regulate the release of acetylcholine and noradrenaline respectively (Chess-Williams, 2002). The receptors present on the parasympathetic nerve are the excitatory M<sub>1</sub> and M<sub>3</sub> receptors, compared to the inhibitory M<sub>2</sub> and M<sub>4</sub> receptors found on the sympathetic nerve endings.

The bladder urothelium is estimated to have up to 40% greater muscarinic receptor density than the detrusor (Hawthorn et al., 2000). Consistent with animal species, the presence of the urothelium in isolated human bladder strip experiments was found to exert an inhibitory effect on cholinergic contractile responses of the underlying detrusor muscle (Chaiyaprasithi et al., 2003). This inhibition has been attributed to the release of an unknown factor from the urothelium, termed urothelial-derived inhibitory factor (UDIF) (Hawthorn et al., 2000). UDIF will be discussed in more detail in later sections.

Acetylcholine also influences sensory nerves. A study by Daly and colleagues (2010) found stimulating muscarinic receptors or inhibiting cholinesterase activity significantly attenuated mechanosensitivity in the bladder. While muscarinic receptor stimulation enhances contraction of the bladder, muscarinic antagonists have been used clinically with success to treat overactive bladder. Commonly termed anticholinergics or antimuscarinics, their mechanism of action is to competitively antagonise muscarinic receptors on the bladder and also potentially inhibit

urothelial sensory receptors and decrease afferent nerve activity (Marinkovic et al., 2012, Yamaguchi et al., 2009).

## **NICOTINIC RECEPTORS**

Compared to muscarinic receptors, there is little known regarding the effect of acetylcholine on nicotinic receptors in the bladder. The urothelium expresses multiple nicotinic receptors, classified by either an  $\alpha$  or  $\beta$  subunit, forming either neuronal or muscular nicotinic receptors (Beckel et al., 2006). Recent investigation into two subtypes of nicotinic receptors,  $\alpha 3$  and  $\alpha 7$ , shows that with stimulation they aid to excite by influencing calcium influx and inhibit neurotransmitter release respectively (Beckel and Birder, 2012).

The external urethral sphincter is comprised of striated muscle, which contracts during bladder filling. This contraction is mediated through the release of acetylcholine from the somatic nerves acting upon the nicotinic receptors present in the urethral sphincter (Andersson and Michel, 2011, Fowler et al., 2008).

## **ATP AND PURINERGIC RECEPTORS**

Adenosine-5'-triphosphate (ATP) was first implicated as the mediator for the NANC contractions from the observation that parasympathetic nerves induce atropine-resistant contractions of the bladder (Burnstock et al., 1972). In addition to being co-released in vesicles with acetylcholine from parasympathetic nerves, ATP has shown to be released from the urothelium in response to stretch (Ferguson et al., 1997, de Groat and Yoshimura, 2001). Ferguson et al. (1997) suggested that the mechanism of ATP release is non-vesicular from three sources of evidence: that the absence of extracellular calcium potentiates ATP release, tetrodotoxin had no effect on electrically induced ATP release and urothelial cells do not possess secretory granules as in sensory nerves (Dixon and Gilpin, 1987). Furthermore, it appears that distension-induced ATP release is mediated by an increase in intracellular  $\text{Ca}^{2+}$ . Firstly, the addition of capsaicin to rabbit urothelium evokes ATP release without distension; stretch-induced ATP release is attenuated by the  $\text{Ca}^{2+}$ -channel blocker nifedipine and finally, distension of urothelial cells with hypotonic solutions also increase intracellular  $\text{Ca}^{2+}$  (Dunning-Davies et al., 2013, Wu et al., 2011)

ATP acts on two groups of purinergic receptors, an ion channel family P2X and a G-protein-coupled receptor family P2Y (Ralevic and Burnstock, 1998). Seven P2X (P2X<sub>1</sub> – P2X<sub>7</sub>) and eight P2Y (P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>8</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub> and P2Y<sub>13</sub>) receptor subtypes have been identified and cloned (Burnstock, 2006, Shaver, 2001).

Purinergic receptors are found in numerous locations of the bladder, and have been suggested to play an important role in regulating normal bladder function. In both the human and rat bladder, P2X<sub>1</sub> receptors have been found in the detrusor muscle, while P2X<sub>3</sub> receptors are found on the afferent nerves (Elneil et al., 2001, Birder et al., 2004, Wang et al., 2005). These receptors function respectively to convey information regarding bladder filling and storage, and have been implicated in nociception from tissue damage and overfilling (Cook and McCleskey, 2000). Furthermore, P2X<sub>3</sub> receptor knockout mice exhibit decreased voiding frequency, pain response and increased bladder capacity with normal filling pressures, which indicate a role for this receptor in peripheral pain responses and afferent pathways controlling bladder reflexes (Cockayne et al., 2000)

In the urothelium, the entire P2X receptor family and the P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>4</sub> subunits are expressed, however the functional role of these receptors are still under examination (Birder et al., 2004, Sun and Chai, 2004, Elneil et al., 2001).

As previously mentioned, there is evidence that ATP is in part responsible for the NANC contractions of the detrusor muscle, mediated by the P2X receptors (Burnstock, 2009). Bladders from patients with a range of lower urinary tract disorders have observed increases in P2X receptor subtypes and a consequent change in nerve mediated contractions, suggesting a role of ATP in the symptoms presented in these patients (Andersson and Arner, 2004, O'Reilly et al., 2002).

ATP is also released from the urothelium in response to cellular stretch, electrical stimulation and osmotic stress (Ferguson et al., 1997, Vlaskovska et al., 2001, Knight et al., 2002). This urothelial-derived ATP acts on the surrounding structures in both an autocrine and paracrine manner. The ATP released from the urothelium acts as a trigger for discoidal/fusiform vesicle exocytosis through signalling at the P2X<sub>2</sub>, P2X<sub>3</sub> and possibly P2Y receptors present on the

urothelial membrane (Truschel et al., 2002, Wang et al., 2005). Urothelial derived ATP release acts in a paracrine manner on the P2X<sub>3</sub> receptors on bladder afferent nerves (Ferguson et al., 1997, Vlaskovska et al., 2001). The stimulated nerve in turn conveys information regarding the degree of bladder fullness and distension to the CNS (Vlaskovska et al., 2001, Wang et al., 2005). It has been suggested that myofibroblasts situated in the suburothelium may have a role in coupling urothelial-derived ATP to afferent excitation, due to the presence of P2Y receptors and their positioning between the urothelium and afferent nerves (Wu et al., 2004).

After release from parasympathetic nerves or the urothelium, ATP is rapidly converted by endonucleotidases to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine (Lazarowski et al., 2003). Dunning-Davies et al. (2013) found that adenosine acts as a potent inhibitor of stretch-induced ATP release, via stimulation of the adenosine-A1 receptor, suggested being due to a reduction in intracellular Ca<sup>2+</sup> levels via activation of ion channels. The presence of the four adenosine receptors A1, A2a, A2b and A3 in the urothelium was confirmed by Yu et al (2006), who also proposed that adenosine is released from the urothelium and may play a role in exocytosis in the umbrella cells.

## **NORADRENALINE AND ADRENERGIC RECEPTORS**

As previously described, the release of noradrenaline from sympathetic nerves induces bladder relaxation. An inhibitory response of bladder smooth muscle and parasympathetic ganglia was observed through catecholamines administered externally and when released endogenously by electrical stimulation of cat hypogastric nerve in 1972 (De Groat and Saum, 1972). Bladder filling is associated with the cyclical relaxation of the detrusor muscle, and the contraction of the bladder neck and urethra to prevent involuntary micturition, with these actions mediated through noradrenaline acting on the various adrenergic G-protein coupled receptors, namely the  $\alpha$ - and  $\beta$ - subunits (Michel and Vrydag, 2006).

## $\alpha$ -ADRENOCEPTORS

The  $\alpha$ -adrenoreceptor (AR) is further characterised by the  $\alpha_1$ - and  $\alpha_2$ - subtypes. The  $\alpha_1$ - AR (coupled with the  $G_q$  protein) is further subtyped into  $\alpha_{1A}$ -,  $\alpha_{1B}$ - and  $\alpha_{1D}$ -, while the  $\alpha_2$ -AR (coupled with the  $G_i$  protein) encompasses the  $\alpha_{2A}$ -,  $\alpha_{2B}$ - and  $\alpha_{2C}$ -family (Bylund, 1988).

The presence of  $\alpha_1$  receptors have been confirmed in the bladder of mice, rats, monkeys and humans (Michel and Vrydag, 2006). In the human bladder, it was found that stimulating these receptors produce a contractile effect, mediated by the phospholipase C complex (**Figure 1.10**) (Goepel et al., 1997). The localisation of these receptor mRNAs, particularly the  $\alpha_{1A}$  and  $\alpha_{1D}$  (majority) subtypes, in the bladder trigone and base correlates with their function of preventing bladder content leakage (Malloy et al., 1998).

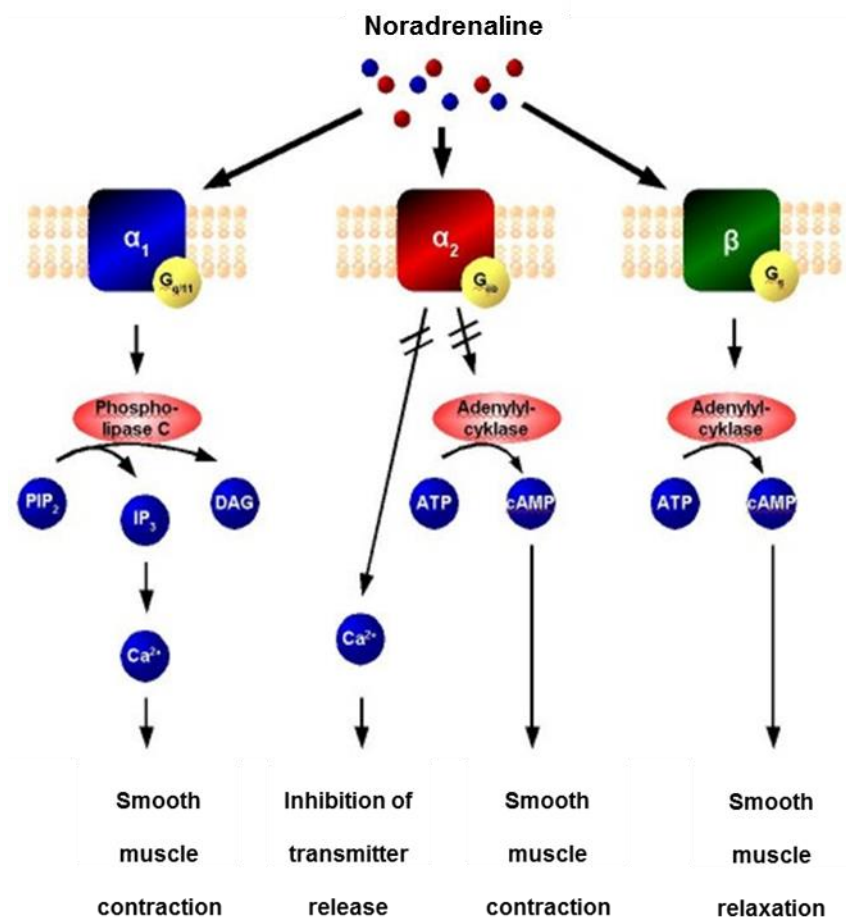


Figure 1.10: Effects of noradrenaline on the  $\alpha_1$ -,  $\alpha_2$ - and  $\beta$ -adrenoceptors.  $\alpha_1$ -ARs initiate smooth muscle contraction coupled to the  $G_q$  protein,  $\alpha_2$ -ARs inhibit neurotransmitter release and initiate smooth muscle contraction coupled to the  $G_i$  protein while the  $\beta$ -ARs initiate smooth muscle relaxation coupled to the  $G_s$  protein.



Radioligand binding studies have confirmed the presence of  $\alpha_2$ -AR coupled to the  $G_i$  protein in the urethra, detrusor and bladder base/neck of rabbits, pigs and humans (Goepel et al., 1997, Levin et al., 1988, Andersson et al., 1984). Despite their presence in the bladder, it has been confirmed that these receptors do not mediate contraction in the rabbit, guinea-pig and human detrusor (Ueda et al., 1984, Kunisawa et al., 1985, Gillespie, 2004). The prejunctional inhibition of post-ganglionic sympathetic and parasympathetic neurotransmitter release (**Figure 1.10**), and direct inhibition of parasympathetic nerve activity in the bladder as found in rabbits, appears to be the most significant function of these receptors.

### $\beta$ -ADRENOCEPTORS

Noradrenaline is released by sympathetic nerves during bladder filling. This acts upon the  $\beta$ -ARs dispersed throughout the detrusor muscle, which relaxes to accommodate bladder filling.

The presence of  $\beta$ -adrenoceptors has been confirmed within the rat and human bladder, with all three subtypes ( $\beta_1$ ,  $\beta_2$  and  $\beta_3$ ) present within both species (Takeda et al., 1999, Fujimura et al., 1999, Igawa et al., 1999, Li et al., 2003). Through the application of selective  $\beta$  agonists, it has been found that the  $\beta_3$ -AR mediates the relaxation of the detrusor muscle through the stimulation of adenylate cyclase, increasing cAMP and activating PKA (**Figure 1.10**) (Igawa et al., 1999, Igawa et al., 2001, Yamaguchi, 2002).

### NITRIC OXIDE

Nitric oxide (NO) is an important signalling molecule released from the bladder in response to mechanical stretch, chemical stimulation including capsaicin, nicotine and calcium ionophores and neurotransmitters (acetylcholine, noradrenaline and substance P) (de Groat and Yoshimura, 2001, Birder et al., 2002). NO is the neurogenic mediator that causes relaxation of the smooth muscle of the bladder neck and urethra during bladder micturition (Ehrén et al., 1994). Stimulation of the adrenoreceptors in the urinary bladder directly induces detrusor relaxation and causes NO release (Birder et al., 1998), however as the detrusor muscle cells lack soluble

guanylate cyclase (receptors for NO), it is likely that NO-induced relaxation of the detrusor is mediated by the localised myofibroblasts (Smet et al., 1996). NO is also released by the urothelium, proposed to play a role in regulating permeability, excites afferent nerve activity (particularly sensitizing nociceptors) and modulates efferent nerve neurotransmitter release (Mumtaz et al., 2000, Gold et al., 1996).

Nitric oxide synthase (NOS) is the enzyme responsible for the catalysis of NO from L-arginine, localized in nerve fibres of the detrusor muscle in the trigone portion of the bladder and the urethra, as well as the urothelium (Andersson and Persson, 1995). Three isoforms of NO synthase (NOS) have been identified in the lower urinary tract, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), named after the cells in which they were first purified and isolated (de Groat and Yoshimura, 2001, Forstermann et al., 1994). The presence of NOS in preganglionic sympathetic and postganglionic parasympathetic nerves in the lower urinary tract of the rat further indicate a role of NO in the inhibition of NANC induced contractions (McNeill et al., 1992, Mumtaz et al., 2000).

Nitric oxide has been implicated in a range of bladder pathophysiology. nNOS knockout mice suffer from increased urinary frequency, and their bladders were found to be hypertrophic and resistant to relaxation by exogenous and neurogenic NO release (Burnett et al., 1997). In comparison, NO production is upregulated in inflamed and chronically infected (eg. due to bacterial cystitis) bladders, resulting in hyper-reflexivity (Nussler and Billiar, 1993). Measurement of bladder NO has also been used to differentiate urgency relating to inflammation from neurogenic disorders or bladder outlet obstructions (Ehren et al., 1999b).

## **PROSTAGLANDINS**

Prostaglandins play a significant role in controlling urinary bladder motility. The release of eicosanoids by the urothelium, in particular prostaglandin E<sub>2</sub>, was first discovered by Jeremy and colleagues (1987), using samples of human bladder obtained during cystoscopy. It was proposed that prostaglandin E<sub>2</sub> play a role in modulating the tone and contractility of the bladder

detrusor muscle directly during the excitation-contraction coupling and indirectly by influencing neurotransmission (Jeremy et al., 1987).

Prostaglandin E<sub>2</sub> is formed through the metabolism of arachidonic acid to prostaglandin H<sub>2</sub> catalysed by cyclooxygenase enzymes, rapidly converting to prostaglandin E<sub>2</sub>. Both the detrusor muscle and urothelium have been found to release prostaglandin E<sub>2</sub>. The detrusor muscle releases prostaglandin E<sub>2</sub> in response to bladder stretch and electrical field stimulation (Park et al., 1999, Park et al., 1997, Dveksler et al., 1989), while the urothelium releases it in response to stretch, potentiated by obstructive and overactive bladder disorders (Masunaga et al., 2006, Pinna et al., 2000).

Early experiments on the bladder muscle observed that the detrusor muscle, but not the urethra, contracted with addition of prostaglandin E<sub>2</sub> to the tissue (Abrams and Feneley, 1975). Prostaglandin E<sub>2</sub> released from the detrusor and urothelium are thought to contribute to the detrusor basal tone and modulate afferent nerve activity and efferent neurotransmission (Anderson, 1993). It was proposed that prostaglandin E<sub>2</sub> has an indirect effect on bladder emptying, through stimulating vanilloid receptors and the release of tachykinins, thus sensitizing afferent nerves (de Groat and Yoshimura, 2001, Morrison, 1999).

Increased urinary prostaglandins have been linked to the pathophysiology of bladder disorders, including urinary tract infections/inflammation and particularly detrusor overactivity experienced in patients with bladder outlet obstruction and (Wheeler et al., 2002, Schroder et al., 2004).

## **UROTHELIAL-DERIVED INHIBITORY FACTOR**

The presence of the urothelium has an inhibitory effect on contractions of the detrusor muscle. This was observed in experiments investigating the contractions of porcine detrusor with and without the urothelium to muscarinic agonists (Hawthorn et al., 2000). A significant difference in response to carbachol was observed between the intact and denuded tissues, with no change with the addition of inhibitors of the NO pathway, purinergic and adrenergic receptors and potassium channels.

It was concluded that a mediator released from the urothelium, termed the urothelial-derived inhibitory factor (UDIF), is responsible for the inhibition of detrusor muscle. At present, it is unclear what the UDIF is, however mediators including NO, eicosanoids, gamma aminobutyric acid, catecholamines and apamin-sensitive response to endothelium-derived hyperpolarizing factor have been ruled out (Hawthorn et al., 2000).

## 1.2 BLADDER CANCER

Bladder cancer is the most common malignancy of the lower urinary tract, and is the seventh and seventeenth most common worldwide cancer in men and women respectively (Burger et al., 2012). This condition is more common in developed countries, and is predominantly a disease of the elderly, most common in the United States and Western Europe (Ferlay et al., 2010). Bladder cancer is approximately three times more common in males than in females, however it is interesting to note that women, particularly of African-American descent along with the elderly, experience higher mortality rates (Scosyrev et al., 2009).

The prevalence of bladder cancer across the globe, as well as the high recurrence rate and necessity of continual monitoring result in this disease having the highest lifetime treatment costs per patient of all cancers, ranging from \$97 000 - \$187 000 per person in the US (Sievert et al., 2009, Racioppi et al., 2012, Botteman et al., 2003). These costs are attributed to the high rate of return of bladder cancer, and the associated long-term side effects from intravesical therapy accounting for approximately a third of all expenses (Avritscher et al., 2006).

The risk factors for bladder cancer are differentiated into genetic and environmental exposures. As the urothelium lines the urinary tract, it is exposed to potential carcinogens and reactive oxygen species excreted in the urine. The most common and significant etiologic factor implicated in bladder cancer development is tobacco smoking (Hecht, 2006, Hartge et al., 1993, Ji et al., 2005). Patients with recurrent or chronic bladder infections and inflammations, such as the human papillomavirus infection, or iatrogenic effects from pelvic radiation or cyclophosphamide and thiazolidinedione treatment have a higher risk of bladder cancers (Groah et al., 2002, Kleinerman et al., 1995, Sandhu et al., 2006, Travis et al., 1995, Colmers et al., 2012). The risk of bladder cancer in a first-degree relative of a patient is increased two-fold, through the inheritance of a predisposition of oncogenes, epithelial growth factor receptors and susceptibility to potential carcinogens (Burger et al., 2012).

Bladder tumours are either epithelial or non-epithelial in origin, approximately 90% being the former. These include transitional cell carcinoma (TCC) squamous cell carcinomas (SCCs), adenocarcinomas and small cell tumours, while the rarer nonepithelial tumours encompass

sarcomas, pheochromocytomas, melanomas and lymphomas. Transitional cell carcinoma (TCC) is the most common of histological neoplasms, representing over 90% of all bladder cancers (Griffiths, 2012). TCCs refer to malignancies originating from the urothelium and are often termed urothelial cancer. For the interests of this investigation, transitional cell carcinoma characteristics and treatment options will be discussed, being the majority of bladder cancer diagnoses. The majority of bladder cancer patients present with painless haematuria (Griffiths, 2012, Kaufman et al., 2009). Other common presenting symptoms are unexplained urinary frequency and urgency and dysuria during voiding.

## **CLASSIFICATION OF BLADDER CANCER**

Classification of bladder tumours are determined by the degree of metastasis and level of invasion through the bladder wall, often termed the grade and stage. Tumour stage and grade is useful in determining and monitoring patients' risk of recurrence and disease progression. In 2004, the World Health Organisation (WHO) in conjunction with the International Society of Urological Pathology (ISUP) proposed a two-tiered grading system as seen in **Table 1.1** (Gorin et al., 2012, Montironi and Lopez-Beltran, 2005). This system delegates tumours to be of either low or high grade, as well as defining tumours to be either papillomas or papillary urothelial neoplasms of low malignant potential (PUNLMP). Previously, tumours were classified as papillomas or grades 1-3 according to the 1973 WHO classification (Montironi and Lopez-Beltran, 2005).

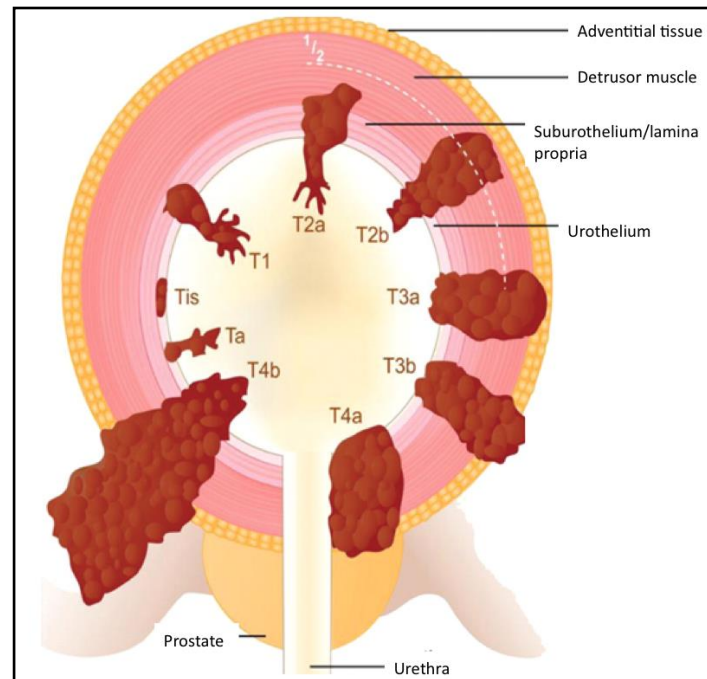
**Table 1.1: WHO/ISUP 2004 bladder tumour grading system (Montironi and Lopez-Beltran, 2005)**

<b>Papilloma</b>	Delicate papillae with cellular organization and cytologic findings identical to normal urothelium
<b>PUNLMP</b>	Occasionally fused papillae, normal cell polarity, uniformly enlarged nuclei, fine chromatin, rare mitotic figures confined to the basal level (encompasses grade 1 tumours from previous 1973 classification)
<b>Low Grade</b>	Fused and branching papillae, minimal cell crowding and loss of polarity, enlarged nuclei of variable size and slightly irregular shape, mild variation of chromatin, inconspicuous nucleoli, mitotic figures occasionally present at any level (includes grade 1 and 2 tumours)
<b>High Grade</b>	Fused and branching papillae, discohesive with loss of cell polarity, enlarged pleomorphic nuclei of variable size, moderate to marked variation of chromatin, multiple nucleoli, frequent mitotic figures at any level (consisting of grade 2 and 3 tumours)

Staging of bladder cancer tumours are based on the level of invasion through the bladder wall, adapted by the 2010 American Joint Commission on Cancer, seen in **Table 1.2** and **Figure 1.11** (Edge and Compton, 2010, Gorin et al., 2012). Non-muscle invasive bladder cancer (NMIBC) encompass Tis (tumours in situ) to the level of T1 (tumours invading to the level of the suburothelium/lamina propria), while muscle invasive tumours (MIBC) include T2 tumours to T4 (Gorin et al., 2012, Griffiths, 2012).

**Table 1.2: Adapted AJCC 2010 staging system of bladder cancer (Edge and Compton, 2010)**

Primary Tumour (T)	
<b>T0</b>	No evidence of tumour
<b>Ta</b>	Papillary tumour confined to the urothelium
<b>Tis</b>	Carcinoma in situ
<b>T1</b>	Invades suburothelium/lamina propria
<b>T2a</b>	Invades superficial muscularis propria
<b>T2b</b>	Invades deep muscularis propria
<b>T3a</b>	Microscopic invasion of perivesical fat
<b>T3b</b>	Macroscopic invasion of perivesical fat
<b>T4a</b>	Invades the prostate, seminal vesicles, uterus, or vagina
<b>T4b</b>	Invades the pelvic or abdominal wall



**Figure 1: Bladder cancer staging (Tis – T4b)**

Bladder cancer has a high propensity to return. Prognostic factors, including age, gender, tumour grade, stage and number must be taken in to account to determine the most appropriate adjuvant treatment. Sylvester and colleagues (Sylvester et al., 2006) designed a number of nomograms from analysis of 2596 NMIBC patients included in seven European Organization for Research and Treatment of Cancer trials. These diagrams allow the determination of patient risk of recurrence and progression post one and five years transurethral resection (TUR), which in turn sheds light on the most appropriate adjuvant treatment and protocol. At present, it has been estimated that up to 90% of all patients will experience recurrence of tumours following transurethral resection over their lifetime (Herr, 2000).

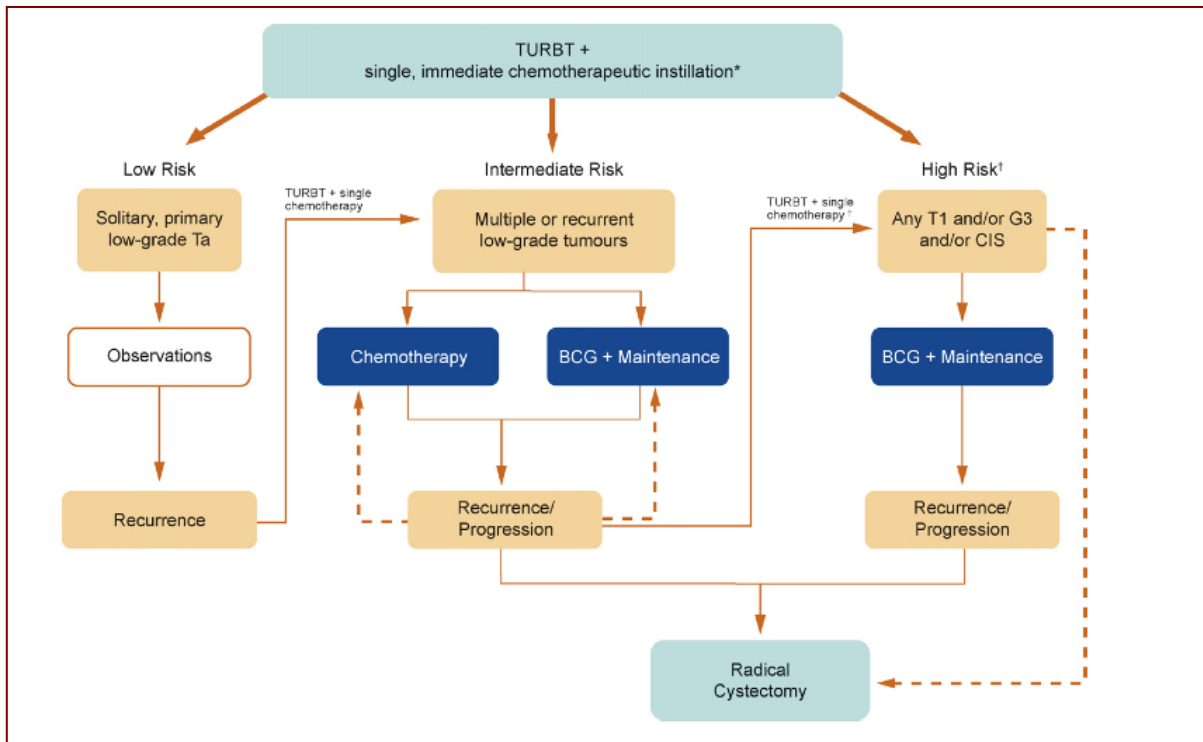


## DIAGNOSIS AND TREATMENT

Bladder cancer is identified with a range of diagnostic tests. Urine cytology and molecular tests are used to investigate the presence of exfoliated cancer cells and soluble or cell-associated markers (Babjuk et al., 2008, Lokeshwar et al., 2005). Visualizing tumours is necessary for tumour diagnosis, performed by intravenous urography or the more commonly used cystoscopy.

At presentation, between 70-80% of transitional cell carcinomas are diagnosed as superficial or non-muscle invasive bladder cancers (NMIBC), referring to carcinomas that are restricted to the bladder urothelium and suburothelium/lamina propria (Shelley et al., 2011b, Hendricksen and Witjes, 2007). Bladder tumours have a high predisposition to return, with 45% of patients having tumour recurrence within the first 12 months of diagnosis (van der Heijden and Witjes, 2009). 10-20% of these recurring tumours will have progressed to be muscle invasive, significantly decreasing their probability of recurrence free survival (Kaufman et al., 2009, Rubben et al., 1988).

The first line of treatment for NMIBC is transurethral resection (dependent on tumour grade); followed by intravesical immunotherapy or chemotherapy, while MIBC patients undergo partial or full cystectomy. **Figure 1.12** depicts an algorithm proposed by the International Bladder Cancer Group for the general treatment and management of non-muscle invasive bladder cancer.



**Figure 1.12: Algorithm for the treatment and management of non-muscle invasive bladder cancer proposed by the International Bladder Cancer Group.** TURBT – transurethral resection of the bladder tumour; BCG – bacillus Calmette-Guerin therapy; CIS: carcinoma (tumour) in situ (reprinted with permission from Lamm et al., 2008)

## TRANSURETHRAL RESECTION AND PARTIAL/FULL CYSTECTOMY

Transurethral resection (TUR) of bladder tumours is used for both diagnostic and therapeutic purposes. A resectoscope is used to remove the tumour entirely or in fractions, dependent on the size and grade of tumours. Small tumours are removed in entirety with part of the underlying wall, while large tumours are resected in fractions, including the exophytic part of the tumour, the underlying detrusor muscle and edges of the resected area (Vaupel et al., 1989, Babjuk, 2009). Succeeding transurethral resections are considered 2-6 weeks following the initial procedure if the tumour was under-staged/graded or the procedure was incomplete.

For patients with muscle invasive tumours graded between T2 and T4, a partial or full cystectomy is considered to be the gold standard in treatment (Sternberg et al., 2007). The five-year survival rate after cystectomy is up to 65% for patients with low graded tumours, and between 25-35% for T3 and T4 stage tumours (Stein et al., 2001, Ghoneim et al., 1997, Addeo

et al., 2010). For the purposes of this report, further discussion of bladder cancer treatment options will refer to the treatment of non-muscle invasive bladder cancer only.

## **INTRAVESICAL IMMUNOTHERAPY**

Intravesical immunotherapy describes the direct instillation of agents to induce an immune response within the bladder. Since 1976, Bacillus Calmette-Guerin (BCG) therapy has been used for the management of bladder cancers as an adjuvant therapy post transurethral resection, after preliminary studies on the dog bladder produced marked inflammatory and immune reactions (Bloomberg et al., 1975, Schellhammer et al., 1975). BCG therapy involves direct instillation of tuberculosis bacteria, with a dose dependent on the strain of virus (Gontero et al., 2010). The most widely used strains in North American and Europe are the BCG Connaught and BCG Tice, with the Connaught strain having significantly improved recurrence-free survival rate for patients with NMIBC compared to the Tice strain (Rentsch et al., 2014).

The cytotoxic mechanism of BCG on cancer cells is still not fully understood, but it is thought that fibronectin-binding proteins on the surface of the BCG bacteria bind to urothelial receptors and initiate intracellular signalling to stimulate anti-tumour activity (Bohle and Brandau, 2003, Okeke et al., 2005). Limitations to this treatment are the systemic toxicity and local urological side effects including cystitis, increased frequency and haematuria (Babjuk et al., 2012). Systemic toxicity manifesting in side effects is less common in patients undergoing BCG therapy, but include fever, myalgia and nausea, as well as rare cases of BCG infection progressing to multiple organ failure and death.

Numerous studies within the literature have confirmed that BCG post-TUR is superior in limiting tumour recurrences compared with TUR alone (Shelley et al., 2004). Conflicting evidence is reported in randomised trials comparing intravesical BCG with intravesical chemotherapy in terms of recurrence, disease progression and overall survival. When comparing to mitomycin C, the first line chemotherapeutic agent used in bladder cancer treatment, a meta-analysis by Shelley et al. (2004) suggested that BCG was the superior treatment in preventing tumour recurrence in high-risk patients, although Bohle and colleagues (2003) reported no significant

difference in intermediate risk patients. Other agents investigated included doxorubicin and epirubicin which are also commonly used in the treatment of NMIBC. In comparison to BCG, both agents were inferior in preventing or delaying tumour recurrence, however there is limited evidence to suggest that BCG improves the rate of tumour progression and overall survival (Shelley et al., 2010, Khanna et al., 1987, Lamm et al., 1991, Hinotsu et al., 2006, van der Meijden et al., 2001).

While the evidence suggests intravesical BCG is the most favourable treatment, it is in some cases an inappropriate option. BCG for patients with tumours with a low risk of progression is considered to be an overtreatment, and the risk of systemic toxicity is significantly higher with BCG compared to intravesical chemotherapy. A dropout rate of 20.3% was seen in a cohort of 487 patients in a 36-month treatment program using BCG, due to the local and/or systemic side effects (van der Meijden et al., 2003), thus requiring alternate treatment plans. Similarly, up to a third of patients with NMIBC will not respond to BCG therapy, and it has been observed that up to 40% of patients who initially respond to BCG therapy, fail with recurring tumours, thus evolving to being BCG-refractory (Herr and Dalbagni, 2003, Herr et al., 1989, Herr, 2005, Witjes, 2006).

Since July 2012 the worldwide production of BCG has struggled to meet demands, after mould compromised the BCG Connaught strain produced by Sanofi Pasteur (Lyon, France) after a flood in the manufacturing plant in Toronto, Canada (Mostafid et al., 2015). This facility was the sole producer of ImmuCyst (BCG Connaught), the market leader of BCG products in many countries, including the UK and United States. The consequent replacement market leader of BCG, OncoTICE (a Tice strain of BCG) manufactured by Merck Sharp & Dohme Ltd (Hertfordshire, UK), announced that it expected severely reduced OncoTICE supplies throughout 2015 due to a combination of increased demand and manufacturing issues (Mostafid et al., 2015). Furthermore, the doses of BCG used for NMIBC treatment are immense with comparison to the amount required for a BCG tuberculosis vaccine, with a 6-instillation course for bladder cancer patients being sufficient to vaccinate 10,000-100,000 people. For this reason, alternative treatments and management plans for NMIBC patients are urgently needed.

## **INTRAVESICAL CHEMOTHERAPY**

Intravesical chemotherapy for non-muscle invasive bladder cancers involves direct instillation of the chemotherapeutic agents into the bladder. Frequently used drugs include doxorubicin, epirubicin, mitomycin C and gemcitabine. For patients with high risk NMIBC, there is a general consensus that intravesical chemotherapy remains the most favourable second-line treatment for BCG refractory patients. Intravesical chemotherapy for patients with low to intermediate risk features such as multifocal or low grade Ta tumours is often considered the most favourable first line treatment (Koya et al., 2006). Regimes for intravesical chemotherapy include immediate and adjuvant treatments, with an optimal protocol of duration and frequency post-TUR yet to be determined (Sylvester et al., 2008).

### **IMMEDIATE POST-TUR CHEMOTHERAPY**

As non-muscle invasive tumours recur frequently and often progress to muscle invasive neoplasms even after TUR, adjuvant therapy is considered for all patients. A meta-analysis of seven-randomised trials showed that an instillation of chemotherapy (epirubicin or mitomycin C) within 24 hours of TUR for NMIBC significantly reduced the risk of recurrence of Ta/T1 tumours (Babjuk et al., 2012, Sylvester et al., 2004). This type of adjuvant therapy is thought to destroy residual and circulating tumour cells at the resection site. As chemotherapeutics generally have a high molecular weight and the presence of the protective GAG and uroplakin layer on the urothelium, there is little risk of absorption and thus systemic toxicity. Due to this, they can be instilled directly post-TUR.

An initial randomised study in patients of Ta/T1 tumours comparing an immediate instillation of epirubicin to water post-TUR found that the interval to recurrence was significantly better and in a two-year follow up the recurrence rate was halved in the epirubicin group (Oosterlinck et al., 1993). Similarly, a meta-analysis reported by Sylvester et al (2004) concurred with these results, finding a 39% decrease in the chance of recurrence with a single post-operative chemotherapeutic dose, with only mild and transient toxicity. From this data, a single instillation

of chemotherapeutic agent immediately post-TUR appears to be the best option for all patients with NMIBC, however further treatment is required for patients with high-risk tumours.

#### ADJUNCT INTRAVESICAL CHEMOTHERAPY

Intravesical chemotherapy is considered as an alternate treatment to Bacillus Calmette-Guerin immunotherapy for superficial bladder cancer, or as an adjunct therapy after refractory BCG. Chemotherapeutic drugs including mitomycin C, thiotepa, epirubicin and doxorubicin have been investigated in intravesical therapy. As discussed previously, the literature suggests that none of these drugs are more effective compared to BCG in treating NMIBC. However, as alternate treatments are required in refractory BCG or low risk NMIBC patients, investigation of chemotherapies is necessary to provide an appropriate second-line treatment. Significant adverse effects have been reported following intravesical instillation of the aforementioned drugs, including chemical cystitis, dysuria, increased frequency and urgency, as well as bladder toxicity (Koya et al., 2006, Thrasher and Crawford, 1992).

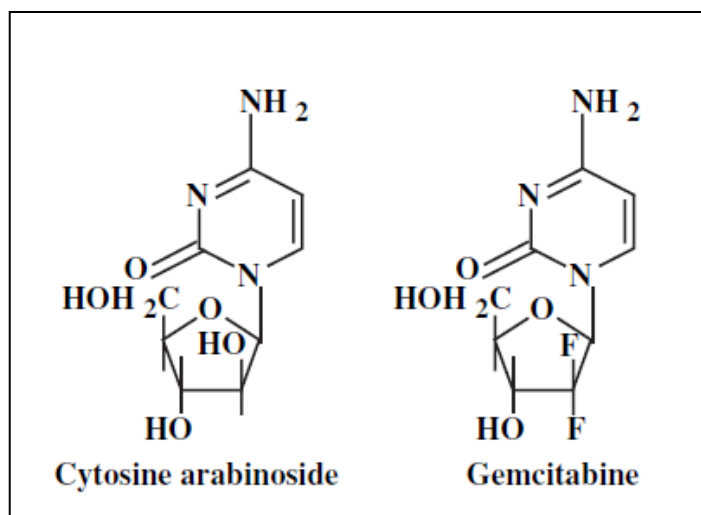
These chemotherapeutics have been tried and tested on NMIBC and BCG-refractory patients, producing limited efficacy and poor toxicity profiles, which has led to the investigation of other chemotherapeutic agents. Gemcitabine, a prodrug chemotherapeutic, is FDA approved and used in the first line treatment in other cancers including, breast, ovarian, small cell lung cancer and pancreatic cancer (Lee, 2009). Although limited, reviews of the literature found that gemcitabine showed positive activity against metastatic bladder cancer, with an improved toxicity profile in comparison to commonly used MVAC (methotrexate, vinblastine, doxorubicin and cisplatin) treatment, mitomycin C and doxorubicin (Shelley et al., 2011a). Discussion of gemcitabine, its metabolism and methods of action will aim to describe its pertinence as an ideal chemotherapeutic for non-muscle invasive bladder cancer.

## 1.3 GEMCITABINE

Gemcitabine (GEM, 2', 2' -difluoro 2' deoxycytidine, dFdC) is a pyrimidine analogue classified as a prodrug (Mini et al., 2006). It is commonly used both singularly and in combination with other anti-neoplasm therapies for the treatment of pancreatic, breast, ovarian, lung and bladder cancer, due to its lack of cross-resistance with other anti-cancer agents (Candelaria et al., 2010, Lee, 2009). Originally investigated as an anti-viral treatment, it was found to have potent anti-cancer activity and a wide spectrum of antitumor properties in 1990 through the inhibition of human leukaemia cells (Hertel et al., 1990). Gemcitabine has a low molecular weight of 299D, compared to that of mitomycin C and doxorubicin, being 389D and 589D respectively (Gontero and Frea, 2006). This may allow it to more effectively penetrate the urothelium, while limiting systemic absorption.

### STRUCTURE AND UPTAKE

Gemcitabine is an analogue of cytosine arabinoside (Ara-C), differing structurally at position 2' with a fluorine substitute in the furanose ring, as shown in **Figure 1.13**. Like Ara-C, gemcitabine requires uptake into the cell and consequent phosphorylation to form its active metabolites. While the intracellular phosphorylated form of Ara-C competes with deoxycytidine triphosphate (dCTP) in inhibiting DNA polymerase activity and acts as a substrate in DNA synthesis (Heinemann et al., 1988), gemcitabine has multiple intracellular targets reflective in its wide antitumour-spectrum (Mini et al., 2006, Candelaria et al., 2010, Hertel et al., 1990).



**Figure 1.13: Structure of Cytosine arabinoside and Gemcitabine (reprinted with permission from Mini et al., 2006).**

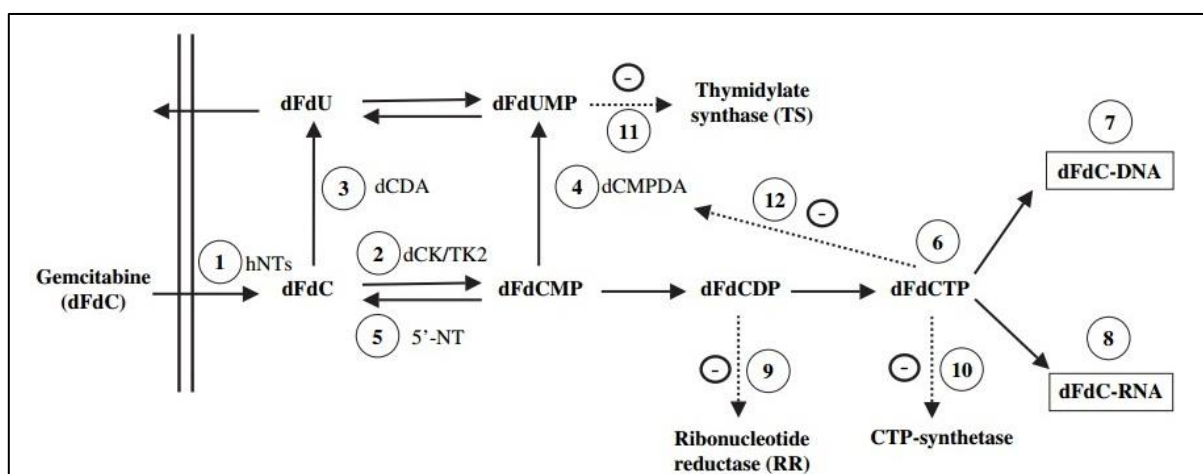
Gemcitabine moves through the cellular membrane via nucleoside transporters (NT) located in the plasma membrane. Human NTs are either concentrative (sodium dependent, hCNTs) or equilibrative (sodium independent, hENTs) type, both of which are involved in gemcitabine uptake. Specifically, gemcitabine acts as a substrate for five of the hNTs, including hENT1, hENT2, hCNT1, hCNT2 and hCNT3 (Mackey et al., 1998, Mabel W. L. Ritzel, 2001). While equilibrative NTs appear to be widely distributed between cell and tissue types, concentrative NTs are limited to cells including intestinal and renal epithelia, liver and leukemic cells (Mackey et al., 1998, Belt et al., 1993). It has been reported that hENT1s have the greatest affinity for gemcitabine, and hENT1 deficient cells are highly resistant to this pyrimidine analogue (Mackey et al., 1998).

## **METABOLISM AND MECHANISM OF ACTION**

Once inside the cell, gemcitabine is phosphorylated by deoxycytidine kinase or thymidine kinase 2 to gemcitabine monophosphate (dFdCMP) (Mackey et al., 1998, Mini et al., 2006). This substrate is then converted to its active forms of gemcitabine 5'-diphosphate (dFdCDP) and triphosphate (dFdCTP), or deaminated to form 2'-deoxy-2'-difluorodeoxyuridine monophosphate (dFUMP) by deoxycytidine monophosphate deaminase (dCMPDA), as shown in **Figure 1.14** (van



der Wilt et al., 2000, Cerqueira et al., 2007, Giovannetti et al., 2007, Heinemann et al., 1992). These metabolites are therapeutically active agents that act by differing mechanisms to induce cell apoptosis and inhibit normal cellular processes. dFdCDP acts by inhibiting ribonucleotide reductase (RR) (Cerqueira et al., 2007), thus hindering the reaction that forms DNA monomers required for eventual cellular synthesis and repair (Baker et al., 1991). Inhibition of this enzyme by dFdCDP depletes intracellular deoxycytidine triphosphate (dCTP), a nucleoside triphosphate used in DNA synthesis, which in turn inhibits dCK, resulting in a more efficient phosphorylation of gemcitabine (Heinemann et al., 1990, Mini et al., 2006).



**Figure 1.14:** Intracellular transport, metabolism and mechanism of action of gemcitabine. Gemcitabine (dFdC) is transported into the cell (1) via human nucleoside transporters (hNT) where it is either phosphorylated (2) by deoxycytidine kinase or thymidine kinase 2 (dCK/TK2) to form gemcitabine monophosphate (dFdCMP), or deaminated (3) to form dFdU. Gemcitabine monophosphate is either deaminated (4) to dFdUMP, dephosphorylated (5) by 5'-nucleotidase, or phosphorylated to its active forms of gemcitabine diphosphate and gemcitabine triphosphate (6). This triphosphorylated form of gemcitabine is incorporated into the cell's DNA (7), RNA (8) and inhibits CTP-synthetase (10), and acts as a self-potentiator by inhibiting the enzyme dCMPDA (12). The diphosphate form of gemcitabine acts to inhibit ribonucleotide reductase (RR) (9). Finally, the presence of dFdUMP acts to inhibit thymidylate synthase (11) (reprinted with permission from Mini et al., 2006).

In comparison, dFdCTP has multiple intracellular effects, as seen in **Figure 1.14**. It is responsible for the self-potentiating characteristic of gemcitabine, as its presence inhibits the action of dCMP deaminase in deactivating dFdCMP, allowing for increased formation of the active phosphorylated forms of gemcitabine (Mini et al., 2006). In the hamster ovary, exposure

to gemcitabine was found to reduce cellular CTP and dCTP concentrations, indicating a block to CTP-synthetase by dFdCTP (Heinemann et al., 1995). The active form of gemcitabine also works to inhibit DNA polymerase, the enzyme that synthesises DNA strands from their nucleotide composites, as well as being incorporated into DNA (Cerqueira et al., 2007, Gandhi and Plunkett, 1990).

Using in vitro models, (Huang et al., 1991) found that dFdCTP competed directly with dCTP for incorporation into the C sites of the growing DNA chain. The placement of this active metabolite at a non-terminal position on the forming strand leads to the termination of chain lengthening, as well as preventing detection and repair by DNA repair enzymes (Gandhi et al., 1996, Candelaria et al., 2010). Inhibition of RNR by dFdCDP works in synergy with the incorporation of dFdCTP into DNA by reducing the competing deoxyribonucleotide molecule availability.

It is unclear as to how the incorporation of gemcitabine into DNA leads to bladder cancer cell death. The presence of dFdCTP in DNA strands has been suggested to promote cell apoptosis in breast and lung cancer by increasing the frequency of collisions of topoisomerase cleavage complexes causing strand breakages (Pourquier et al., 2002). In human lung-cancer cell lines, the apoptotic-inducing gene p53 was upregulated in a concentration-dependent manner after treatment with gemcitabine (Tolis et al., 1999), while accumulation of capsase-3, the apoptotic regulatory cell protein, was measured within pancreatic cancer cell lines (Chandler et al., 2004). It appears in bladder cancer cell lines that gemcitabine induces cell cycle arrest in the G1 phase, and that cell lines with the gene p53 are inherently more sensitive to the cytotoxic effects of gemcitabine (da Silva et al., 2010). It has been reported that dFdCTP is also incorporated into RNA in a concentration-dependent manner, however the effect on cell function is unknown (Ruiz van Haperen et al., 1993).

## GEMCITABINE ROLE IN NON-MUSCLE INVASIVE BLADDER CANCER

Phase I studies investigated gemcitabine treatment in patients with superficial TCC refractory to BCG with doses between 500-2000mg in saline, with instillation times of one hour (Dalbagni et al., 2002). Minimal urinary symptoms and systemic absorption was observed, similarly in consequent Phase II studies, resulting in a recommended regime of a 2000mg dose weekly for four to six weeks as an initial treatment (Campodonico et al., 2005, Bartoletti et al., 2005). Refractory BCG patients in Phase II trials had excellent tolerability to gemcitabine, with 50% and 23% achieving a complete and partial response respectively with a 1-year recurrence-free survival at 21% (Dalbagni et al., 2006). The low molecular weight, high lipid solubility and high plasma clearance of gemcitabine, as well as its excellent safety and toxicology profile make it an ideal intravesical chemotherapeutic for NMIBC (Laufer et al., 2003).

Within the literature, there are limited comparisons of efficacy between gemcitabine and other intravesical agents commonly used in NMIBC treatment. A summary of these can be seen in **Table 1.3**, adapted from a Cochrane Review as published in 2012 (Jones et al., 2012). An immediate, single post-TUR instillation of gemcitabine was found to have no significant superiority to placebo, with overall recurrence rates of 35.5% and 36.3% respectively (Bohle et al., 2009). In comparison, intravesical gemcitabine therapy shows a favourable efficacy and toxicity profile against mitomycin C (Addeo et al., 2010). Notably, the recurrence rate post initial and maintenance therapy was 28% for gemcitabine and 39% for mitomycin C, with a smaller proportion of patients experiencing adverse effects in the gemcitabine group (38.8% compared to 72.2% for mitomycin C). BCG therapy had a superior recurrence rate to that of gemcitabine treatment post TUR, (28.1% to 53.1%), however in BCG-refractory patients, gemcitabine showed a significantly ( $p < 0.05$ ) improved recurrence profile (52.5% vs 87.5%) (Porena et al., 2010, Di Lorenzo et al., 2010).

It appears from the literature that gemcitabine is a viable treatment option for NMIBC when BCG is not an appropriate option, or for BCG refractory patients. Initial comparisons to mitomycin C display superior efficacy and toxicity profile of gemcitabine, however further investigation is required against other commonly used chemotherapeutics including doxorubicin and epirubicin.

**Table 1.3: Summary of Randomised Trials with Intravesical Gemcitabine (NR – not reported)**

Study	Patients/ Tumour Grade	Interventions	Recurrence Rate	Recurrence Free Survival (months)	% Patient Progression by stage	Incidence of Adverse Effects
<b>Bohle et al. 2009</b>	248 eligible primary or recurrent Ta-T1, G1-3	Single gemcitabine instillation post TUR vs Placebo	38.7%  37.1%	37.2 (median)  40.2	2.4%  0.8%	NR
<b>Addeo et al. 2010</b>	109 eligible recurrent Ta-T1, G1-3	Gemcitabine vs Mitomycin C	28%  39%	NR  15	11.1%  18.2%	38.8%  72.2%
<b>Porena et al. 2010</b>	64 Ta-T1, G1-3 and/or CIS	Gemcitabine vs BCG	53.1%  28.1%	25.6 (mean)  39.4	0%  0%	NR  NR
<b>Lorenzo et al. 2010</b>	80 eligible Ta-T1, low-high grade BCG-refractory	Gemcitabine vs BCG	52.5%  87.5%	3.9  3.1	33%  37.5%	37.5% (grade2-3) 40%

## **LIMITING FACTORS OF INTRAVESICAL TREATMENT**

The adverse effects that are commonly associated with immuno- and chemotherapy singularly and in combination with hyperthermia therapy are a significant limiting factor in intravesical treatment for NMIBC. Approximately 20% of patients cease BCG immunotherapy due to the local and systemic side effects, while urological side effects are commonly reported in intravesical chemotherapy patients (van der Meijden et al., 2003). While there are numerous studies investigating the toxicity profiles of various treatments on the cancer cells, the effects of these intravesical agents on the normal bladder tissue function remain poorly explored within the literature.

Intravesical immunotherapy using *Bacillus Calmette-Guerin* has been proven to be superior to chemotherapy in reducing the risk of recurrence of bladder cancer (Koya et al., 2006, Shelley et al., 2004). However, BCG therapy is associated with more local and systemic side effects compared to other intravesical agents (Sylvester, 2011). Dysuria and increased frequency and urgency are common local side effects, with systemic absorption manifesting as flu-like fevers to fatal sepsis (in rare cases) (Koya et al., 2006). A study of 2602 patients who underwent BCG therapy reported that 95% of all patients exhibited some form of irritative lower urinary tract symptoms (Lamm et al., 1992). As described in a recent Cochrane review (Shang et al., 2011), BCG is associated with significantly more drug-induced cystitis in patients compared to epirubicin treatment, at 54.1% and 31.7% respectively. Additionally, it was reported that 34.1% of patients suffered systemic adverse effects with BCG therapy, including, fever, influenza-like syndrome, nausea, vomiting, BCG lung infection, skin rash and general malaise (Shang et al., 2011)

Intravesical chemotherapy is a commonly used treatment in patients with low to intermediate risk and grade tumours, and as a second line therapy in BCG refractory patients. Due to the high molecular weight of commonly used chemotherapeutics, there is little risk of absorption and consequent systemic toxicity. However, there are often reports of local toxicity including increased frequency and urgency, suprapubic pain and cystitis (Bolenz et al., 2006, Thrasher and Crawford, 1992).

Mitomycin C has potent antitumour activity due to its alkylating properties inhibiting DNA synthesis, and is the first line chemotherapy used for bladder cancer treatment (Rockwell et al., 1982). Commonly reported side effects from intravesical mitomycin C treatment include chemical cystitis, increased frequency and urgency of urination, general discomfort and in some cases contact dermatitis. Chemical cystitis has been reported in up to 40% of patients, in addition to approximately 50% of patients describing dysuria post mitomycin C therapy (Bolenz et al., 2006, Thrasher and Crawford, 1992, Bohle et al., 2003, Au et al., 2001). Allergic reactions have been reported in 5-10% of patients, caused by either direct contact of the drug with or dermatitis due to a delayed hypersensitivity response (Witjes et al., 1998, de Groot and Conemans, 1991, Koya et al., 2006). Another rare side effect is decreased bladder volume and increased contracture of the detrusor muscle post-treatment (Duque and Loughlin, 2000). These changes were observed in rats treated intravesically with mitomycin C, where a significant difference between the bladder volume and compliance of treated and control rats was found, the effects lasting 3-weeks post cessation of therapy (Michielsen et al., 2005).

Doxorubicin and epirubicin are anthracycline antibiotics that act to crosslink tumour DNA, commonly used for treatment of NMIBC (Koya et al., 2006). Epirubicin is a derivative of doxorubicin, both having a molecular mass of 580 kDa, which limits systemic absorption and complications when used for NMIBC treatment. Similarly to mitomycin C, intravesical therapy with these drugs causes local side effects, manifesting as dysuria, increased frequency, urgency and subpubic pain. A study by (Ali-el-Dein et al., 1997) compared instillations of 50mg and 80mg of epirubicin to 50mg of doxorubicin, reported that side effects, namely cystitis and haematuria developed in 15%, 23.5% and 41.7% of patients respectively. A further study reported that 7% of patients out of 829 receiving 50mg of epirubicin in various treatment schedules had to stop treatment due to the severity of cystitis and haematuria, among other local and systemic side effects (Hendricksen et al., 2008). An *in vitro* mouse study comparing doxorubicin and mitomycin C therapy and subsequent tolerance to radiation found that the treated mice exhibited a three-fold increase in micturition frequency, recovering 2-3 weeks post treatment (Post et al., 1995).

In comparison to the aforementioned therapies, gemcitabine treatment for NMIBC appears to have a better toxicity profile and fewer reported side effects (Shelley et al., 2012). A randomized Phase III trial of gemcitabine and mitomycin C reported the incidence of chemical cystitis and dysuria frequency being statistically lower in the gemcitabine group compared to the mitomycin C arm (Addeo et al., 2010). A Phase II trial found 81.3% of patients undergoing weekly 40mg/mL gemcitabine treatment reported no local side effects (Bartoletti et al., 2005). 12% of patients reported urgency during the first instillations, while 2 patients (out of 116) ceased treatment due to ulcerative lesions on the bladder and parosmia. However, a further Phase II trial with the same treatment schedule described 38% of patients reporting Grade 1 dysuria, with two patients (out of 39) requiring antibiotic treatment for a positive urine culture (Gontero et al., 2004).

## **1.4 SUMMARY**

Extensive research has focused on investigating the effects of immunotherapy and chemotherapeutic agents on bladder cancer progression, recurrence and effects at a cellular level on the tumour cells. These studies often report local and systemic side effects, however no studies to date have assessed at a cellular or physiological level the effect of these drugs on the function of normal bladder tissue.

Due to the high recurrence rate and the long-term side effects associated with intravesical therapy, bladder cancer patients have the highest lifetime treatment costs of all cancer patients (Racioppi et al., 2012). Management of the side effects caused by intravesical therapy accounts for approximately a third of all expenses (Avritscher et al., 2006).

These highlight the need for an understanding of the mechanisms of the commonly reported local side effects and thus identify approaches that reduce the incidence and severity of adverse effects. Comprehensive studies of this subject will provide potential mechanisms to treat or prevent the commonly reported side effects, thus improving patient quality of life and minimizing costs.



## **1.5 RESEARCH QUESTIONS AND HYPOTHESIS**

The overall objective of this study was to investigate the changes in bladder function following gemcitabine treatment. Specifically, this study aims to address the following research questions:

1. Does gemcitabine treatment affect the responses of the urinary bladder urothelium, detrusor muscle and intact tissue to purinergic, cholinergic and adrenergic stimulation?
2. Does gemcitabine treatment affect the immediate response to neurogenic stimulation of the detrusor muscle?
3. Does gemcitabine treatment affect basal and stretch induced urothelial mediator release?
4. Does gemcitabine treatment induce the release of inflammatory cytokines?
5. Does gemcitabine treatment affect voiding pattern in mice?

The hypothesis for this study is that gemcitabine treatment will alter the response of the urinary bladder in the aforementioned scenarios.





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## **CHAPTER 2:**

### **GENERAL METHODS**

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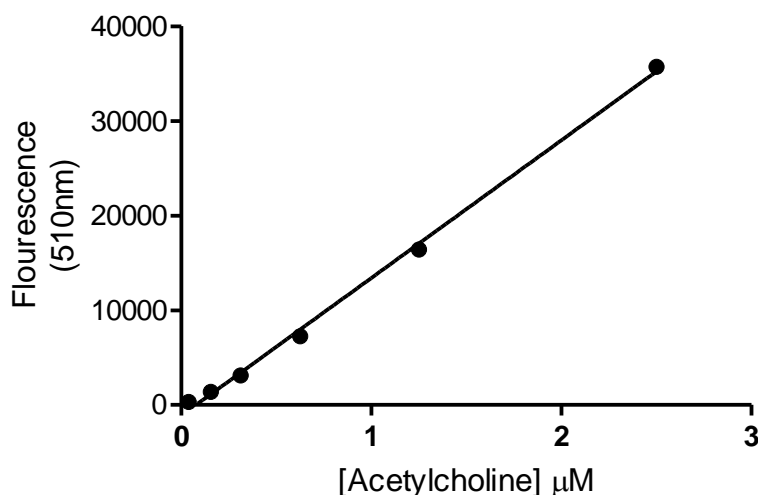


## 2.1 ASSAYS FOR UROTHELIAL MEDIATOR RELEASE

The urothelial mediators ATP, ACh and prostaglandin E<sub>2</sub> collected across all experiments were measured using respective assay kits, according to the product protocols.

### ACETYLCHOLINE ASSAYS

Acetylcholine release was determined using Molecular Probes Amplex Red Acetylcholine/Acetylcholinesterase Assay Kit (A12217). This assay works through acetylcholinesterase (AChE) converting the ACh present in the sample to choline, which is in turn oxidized by choline oxidase to betaine and H<sub>2</sub>O<sub>2</sub>. The presence of horseradish peroxidase allows the reaction between H<sub>2</sub>O<sub>2</sub> and the Amplex Red reagent, causing the production of resorufin. This fluorescent product can then be measured using a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA) with little interference from autofluorescence in samples, due to its close absorption and fluorescence emission maxima (approximately 571nm and 585nm respectively). A standard curve using known acetylcholine concentrations was produced for every plate and used to determine the ACh content in samples, as seen in **Figure 2.1**.

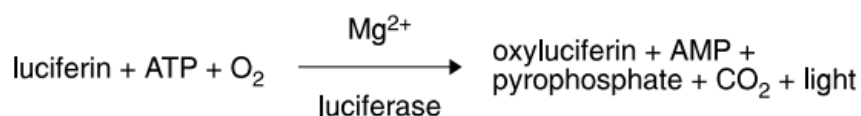


**Figure 2.1: Standard linear regression of fluorescence of samples of known acetylcholine concentrations**

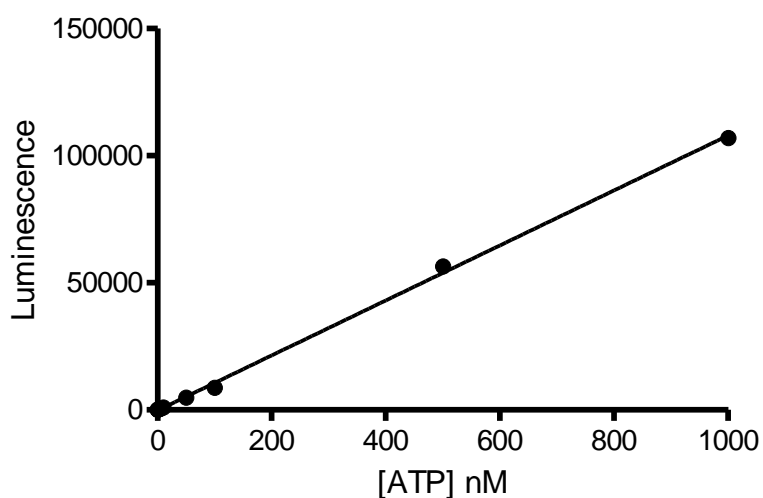
As this assay is based upon choline oxidation, any acetylcholine degraded during sample collection was still measured.

## ADENOSINE TRIPHOSPHATE ASSAYS

ATP release was quantified using the Molecular Probes ATP Determination Kit (A22066). This kit is a bioluminescence assay using recombinant firefly luciferase and its substrate D-luciferin to determine ATP concentrations. The presence of ATP allows the conversion of luciferin to oxyluciferin, producing light as a by-product (emission maximum ~560nm at pH 7.8).



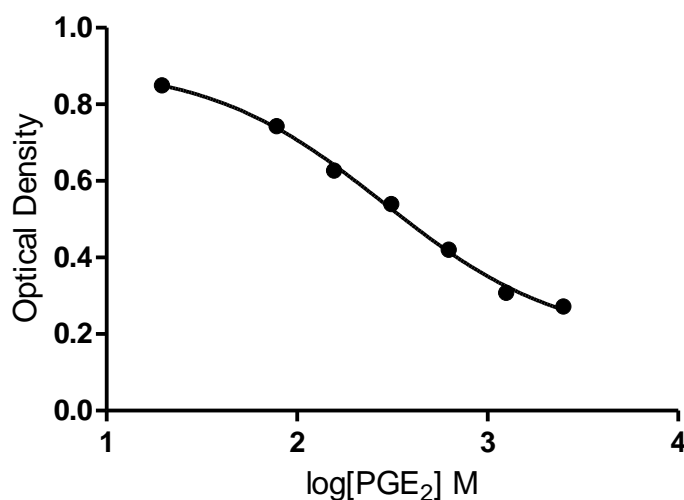
The luminescence of this reaction is measured with a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA), and ATP from unknown samples are determined from a standard curve using known ATP standards, such as in **Figure 2.2**.



**Figure 2.2: Standard linear regression of luminescence readings of known ATP concentrations**

## PROSTAGLANDIN E<sub>2</sub> ASSAYS

Prostaglandin E<sub>2</sub> sample quantification was determined using the R&D Systems Parameter™ prostaglandin E<sub>2</sub> Assay (KGE004B). This assay is based on the sequential competitive binding technique where prostaglandin E<sub>2</sub> in samples compete with horseradish peroxide-labelled prostaglandin E<sub>2</sub> for a limited number of binding sites on a mouse monoclonal antibody. Sampled prostaglandin E<sub>2</sub> binds to the antibody in the first incubation, and with the addition of horseradish peroxide-labelled prostaglandin E<sub>2</sub>, any remaining binding sites are taken. Removal of unbound materials is followed by the addition of a substrate solution to quantify the bound enzyme activity. The intensity of colour of samples, which is inversely proportional to the concentration of prostaglandin E<sub>2</sub>, is measured by a Modulus Microplate Multimode Reader (Turner Biosystems, California, USA) with the absorbance of colour read at 450nm. The concentration of unknown samples was determined by a standard curve using samples of known prostaglandin E<sub>2</sub> concentrations, as shown in **Figure 2.3**.



**Figure 2.3:** Standard curve of absorbance readings of prostaglandin E<sub>2</sub> samples of known concentration



## 2.2 CHEMICALS AND DRUGS

### KREBS SOLUTION

Krebs solution was used for all tissue experiments. The ingredients of this solution are below.

Table 2.1: Krebs solution ingredients

Compound	Compound Formula	Supplier	Catalogue Number	Molarity ( $\mu\text{M}$ ) (in 5L dH <sub>2</sub> O)	Weight (for 5L)
Sodium chloride	NaCl	Sigma Aldrich	S7653	118.41	34.6g
Glucose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	Sigma Aldrich	G0350500	11.65	10.5g
Sodium bicarbonate	NaHCO <sub>3</sub>	Sigma Aldrich	S5761	25.00	10.5g
Potassium chloride	KCl	Sigma Aldrich	P9333	4.56	1.7g
Magnesium sulphate	MgSO <sub>4</sub>	Sigma Aldrich	M7506	2.41	1.45g
Potassium dihydrogen orthophosphate	KH <sub>2</sub> PO <sub>4</sub>	Sigma Aldrich	RES20760-A7	1.18	0.8g
Calcium chloride	CaCl <sub>2</sub>	Sigma Aldrich	21115	1900	9.5mL (1M)

## DRUGS

The drugs used within this project are listed in the table below. Stock solutions were made up in dH<sub>2</sub>O where possible, or in DMSO (Sigma Aldrich), and diluted in either Krebs solution or dH<sub>2</sub>O. Where appropriate, a vehicle control was used to account for any effects of the solvent. Concentrations of each pharmacological agent used within specific experiments are described in the corresponding chapters.

**Table 2.2: Drug compounds, mechanism of action and suppliers**

Compound Name	Compound Formula	Main Action	Supplier	Catalogue Number
<b>α,β-Methyleneadenosine 5'-triphosphate</b>	C <sub>11</sub> H <sub>18</sub> N <sub>5</sub> O <sub>12</sub> P <sub>3</sub>	P2X-purinergic agonist	Sigma Aldrich	M6517
<b>Adenosine 5'-triphosphate salt</b>	C <sub>10</sub> H <sub>14</sub> N <sub>5</sub> Na <sub>2</sub> O <sub>13</sub> P <sub>3</sub>	Purinergic agonist	Sigma Aldrich	A2383
<b>Carbachol</b>	NH <sub>2</sub> COOCH <sub>2</sub> CH <sub>2</sub> N(Cl)(CH <sub>3</sub> ) <sub>3</sub>	Muscarinic agonist	Sigma Aldrich	C4382
<b>Isoprenaline HCl</b>	C <sub>11</sub> H <sub>17</sub> NO <sub>3</sub> -HCl	β-adrenoceptor agonist	Sigma Aldrich	I5627
<b>Atropine</b>	C <sub>17</sub> H <sub>23</sub> NO <sub>3</sub>	Competitive muscarinic antagonist	Sigma Aldrich	A0132
<b>N-Nitro-L-arginine (L-NNA)</b>	C <sub>6</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	Inhibitor of nitric oxide synthase	Sigma Aldrich	N5501
<b>S-(4-Nitrobenzyl)-6-thioinosine (NBMPR)</b>	C <sub>17</sub> H <sub>17</sub> N <sub>5</sub> O <sub>6</sub> S	Inhibitor of equilibrative nucleoside transporters (ENTs)	Sigma Aldrich	N2255
<b>Gemcitabine HCl</b>	C <sub>9</sub> H <sub>11</sub> F <sub>2</sub> N <sub>3</sub> O <sub>4</sub> -HCl	Chemotherapeutic	Sapphire BioScience	A10423
<b>Mitomycin C</b>	C <sub>15</sub> H <sub>18</sub> N <sub>4</sub> O <sub>5</sub>	Chemotherapeutic	Sapphire BioScience	A11435
<b>Tetrodotoxin</b>	C <sub>11</sub> H <sub>17</sub> N <sub>3</sub> O <sub>8</sub>	Selective blocker of Na <sup>+</sup> channels	Sigma Aldrich	T8024

## 2.3 HISTOLOGY

Histological chemistry was performed on various tissues including porcine and murine bladder tissues. Porcine tissues were dissected to an approximate size of 5mm long, 3mm wide and 3mm thick. Murine bladders were approximately 1-2mm thick. All histology processes were performed according to the following protocol.

1. Fixing: Tissues were placed into neutral buffered formalin (10%) and left for 24 hours at 4°C. For whole mouse bladder a catheter was secured in the urethra and formalin was injected into the bladder to gently distend the bladder and then placed in formalin to fix the tissue in a distended state.
2. Processing: The tissues were removed from the formalin and placed in the following solutions:
  - a. 50% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
  - b. 75% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
  - c. 90% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
  - d. 100% ethanol (15 minutes for murine tissue, 30 minutes for porcine tissue),
  - e. 1:1 xylene:ethanol (7 minutes for murine tissue, 15 minutes for porcine tissue),
  - f. 100% xylene (7 minutes for murine tissue, 15 minutes for porcine tissue),
  - g. Fresh 100% xylene (7 minutes for murine tissue, 15 minutes for porcine tissue),
  - h. 1:1 xylene:paraplast at 57°C (15 minutes for murine tissue, 30 minutes for porcine tissue),
  - i. Liquid paraplast at 57°C (15 minutes for murine tissue, 30 minutes for porcine tissue),
  - j. Change paraplast at 57°C (15 minutes for murine tissue, 30 minutes for porcine tissue).

Tissues were positioned in aluminium moulds and completely covered with liquid paraplast. A plastic cassette was placed in the paraplast and left for 12 hours to set.

3. Mounting: Tissue sections were cut at 4-5µm using an Accu-Cut<sup>®</sup> SRM<sup>™</sup> 200 Rotary Microtome (Sakura Finetek Europe B.V., The Netherlands). Sections were placed in a

small water bath at 48°C and then gently positioned on a glass microscope slide and left until dry.

4. Rehydrating: Slides were placed in a heat box for 5-10 minutes and then placed in the following solutions:

- a. 100% xylene for 1 minute,
- b. Fresh 100% xylene for 1 minute,
- c. 1:1 ethanol:xylene for 3 minutes,
- d. 100% ethanol for 3 minutes,
- e. 90% ethanol for 3 minutes,
- f. 75% ethanol for 3 minutes,
- g. 50% ethanol for 3 minutes, and
- h. Distilled water for 3 minutes.

5. Staining: Slides were placed in the following solutions:

- a. Haematoxylin stain (mayers) for 4 minutes
- b. Distilled water for 30 seconds
- c. Acid alcohol (0.5 or 1%) for 30 seconds
- d. Distilled water for 30 seconds
- e. Scott's Blue for 30 seconds
- f. Distilled water for 30 seconds
- g. Eosin stain for 2 minutes
- h. Distilled water for 30 seconds

6. Dehydrating: Stained slides were then placed in the following solutions:

- a. 50% ethanol for 3 minutes
- b. 75% ethanol for 3 minutes
- c. 90% ethanol for 3 minutes
- d. 100 % ethanol for 3 minutes
- e. Fresh 100% xylene for 1 minute

7. Visualisation: Slides were allowed to dry and then visualised under a microscope (Infinity 2, Olympus, Tokyo). Photographs were taken using an Infinity 2 microscope

camera (Olympus, Japan) attached to a computer using Infinity Capture software (version 5.0.2 Lumenera Corporation, Canada).

8. Urothelial thickness was measured using Image J software and were compared between treated and control to evaluate changes. At least seven urothelial measurements were obtained from each bladder section, using a blinded analysis.

All histology solutions and chemicals were Fronine Pathology products obtained from Thermo Fisher Scientific, Taren Point, Australia.

Further methodology specific to studies will be described in the methods sections of the corresponding chapters.

## **2.4 ANALYSIS**

Various statistical tests were used where appropriate and details of analysis are stated in the relevant sections of each Chapter. Statistical differences were considered significant at  $p < 0.05$ . All graphical analyses used in this thesis were performed using GraphPad Prism (Version 5.04 for Windows, Graph Pad Software, San Diego, USA) and all statistical analyses were performed using GraphPad InStat (version 3.06 for Windows, GraphPad Software, San Diego, USA). Specific tests used are described in each of the experimental chapters.





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**CHAPTER 3:**

**EFFECTS OF GEMCITABINE ON CULTURED HUMAN  
UROTHELIAL CELLS**

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### **3.1 INTRODUCTION**

As discussed in Chapter 1, the urothelium plays a significant role in coordinating bladder function. While the urothelium was previously thought to act solely as a distensible barrier protecting underlying tissues from the contents of urine, recent studies have suggested a role in sensory responses to mechanical and chemical stimuli (Birder, 2011). The urothelium responds to stretch during bladder filling by releasing a number of mediators that influence sensory mechanisms and contraction of the underlying detrusor muscle, including ATP, acetylcholine and prostaglandins (Ferguson et al., 1997, Hanna-Mitchell et al., 2007, Tanaka et al., 2011). Given that this layer is in immediate contact with cytotoxic drugs during intravesical treatment, urothelial function is likely affected following treatment and it is the focus of this chapter.

#### **GEMCITABINE PENETRATION THROUGH THE UROTHELIUM**

Intravesical gemcitabine treatment for bladder cancer is localised, in that it sits in direct contact with the urothelium and the tumour. As discussed previously, the urothelium is protected from bacteria, proteins and other small molecules through the presence of the GAG layer (Parsons et al., 1979, Birder, 2011). The low molecular weight of gemcitabine (299D) with comparison to other chemotherapeutics like mitomycin C (389D) and doxorubicin (589D) may enable penetration of the urothelium, but is high enough to prevent significant systemic absorption (Gontero and Frea, 2006). The systemic elimination half-life of gemcitabine is extremely short, within the range of 10-30 minutes. Gemcitabine is extensively deaminated systemically by cytidine deaminase in the liver, kidney and plasma to the uracil metabolite 2',2'-difluorodeoxyuridine (dFdU), which has significantly lower cytotoxicity profiles than its parent compound (Plunkett et al., 1995).

One of the cytotoxic mechanisms of gemcitabine on cancer cells is the induction of enhanced reactive oxygen species (ROS) formation (Donadelli et al., 2011). ROS are produced as by-products of cellular metabolism principally from the mitochondria, but also in response to cytokines, noxious stimuli and bacterial invasion (Ray et al., 2012). Paradoxically, ROS play a role in normal cellular proliferation, can promote carcinogenesis due to oxidative stress, and can

also induce apoptosis of tumour cells (Laurent et al., 2005). Elevated ROS have been detected in almost all cancers, and represent a specific susceptibility of malignant cells to be selectively targeted by pro-oxidant drugs (Laurent et al., 2005, Szatrowski and Nathan, 1991). The enhanced production of ROS can inflict damage to a range of cellular components, including DNA strand breaks or crosslinks, protein damage to amino acid side chains and peptide cleavage and damage to lipid membranes (Halliwell, 2006, Randerath et al., 1996, Stadtman, 1990). Furthermore, it is well reported that excessive production of cellular ROS can induce cell death through apoptosis or necrosis (Sastre et al., 2000, Pelicano et al., 2003, Higuchi, 2003).

Donadelli et al. (2007) found that growth inhibition of pancreatic adenocarcinoma cells by gemcitabine was dependent on ROS production, as there was significantly reduced growth inhibition with the addition of the free radical scavenger N-acetyl-L-cysteine. Additionally, a positive correlation was observed between intrinsic cellular ROS stress and sensitivity to cytotoxicity of gemcitabine in cancer cell lines (Donadelli et al., 2007). Furthermore, it was recently shown by Dalla Pozza et al. (2012) that gemcitabine-induced apoptosis of bladder cancer cell line RT112 is correlated with increased mitochondrial superoxide production, further enhanced by the inhibition of mitochondrial antioxidant properties.

Both pre-clinical and clinical studies have determined that intravesical gemcitabine treatment limits bladder specific toxicity as well as systemic absorption. A study investigating weekly intravesical administration of gemcitabine in rabbits at 25mg/kg and 50mg/kg doses found no significant systemic drug absorption or organ-specific toxicity (Matera et al., 2004). Clinical investigations treating patients with a range of 500-2000mg/50mL have all reported very little to no gemcitabine present within the plasma, with small concentrations of the deaminated metabolite dFdU (0.5-2.7 $\mu$ mol/L) (Dalbagni et al., 2006, De Berardinis et al., 2004, Laufer et al., 2003). The penetration of chemotherapeutics doxorubicin and mitomycin C through the bladder urothelium is reduced 32- and 35-fold respectively from the intravesical dosage (Wientjes et al., 1993, Wientjes et al., 1996). The bladder pharmacokinetics of intravesical gemcitabine have not yet been elucidated, and for this reason, a range of concentrations of gemcitabine was used for treatment on urothelial cells within this study. As superficial bladder cancer has a high propensity to return and progress to invasive tumours, penetration to the sub-layers of the

urothelium is a desirable attribute of chemotherapies. Adjunct therapies such as localised hyperthermia (heat) in combination with chemotherapy have been suggested to increase cellular membrane permeability, alter blood perfusion and enhance the cytotoxicity of chemotherapeutics (Lammers et al., 2011).

## **ADJUNCT THERAPY – HYPERTHERMIA TREATMENT**

The therapeutic benefit of local hyperthermia (temperatures above 37°C) on cancer cells was first recognised in 1952 (Barth et al.). Selawry et al (1957) observed that temporary interruption to the mitotic cycle occurred in cells incubated between 39-40°C, while irreversible heat damage occurred at temperatures above 40.5°C (Rampersaud et al., 2010). Localised hyperthermia therapy for neoplasms has since evolved, and is currently used in conjunction with other therapies, such as chemotherapeutics. One of the challenges in hyperthermia treatment is its ability to effectively deliver localised treatment. The location and structure of the bladder and its constituent cells, and the improved chemosensitivity of urothelial carcinomas with heat makes it an ideal organ for chemohyperthermia treatment.

## **EFFECTS OF HYPERTHERMIA ON THE CANCER CELL**

Tumour cells respond to hyperthermia treatment in multiple ways, promoting tumour necrosis and apoptosis. The effects of hyperthermia on cancer cells are multifactorial, including cytotoxic damage, injury to the surrounding vasculature and an increase in the immune response and thermosensitivity when used in conjunction with chemotherapeutics (Rampersaud et al., 2010). While there is no intrinsic difference in thermal sensitivity between normal and tumour cells, *in vivo* models have found temperatures between 40-44°C to exhibit a tumour cell killing effect (van der Zee, 2002). In comparison, most normal body tissues, including the bladder, are undamaged by one-hour hyperthermia treatment up to temperatures of 44°C (van der Zee, 2002, Fajardo, 1984).

### *CYTOTOXIC EFFECTS*

Hyperthermia causes cytotoxicity in cancer cells in phases. Direct heat exposure causes initial reversible metabolic arrest, followed by irreversible cytotoxicity caused by protein denaturation and cell membrane disruption (Dewey et al., 1971, Westra and Dewey, 1971, Rampersaud et al., 2010). Additionally, at temperatures above 42°C, RNA and DNA synthesis are diminished, the latter remaining inhibited after treatment due to the heat-induced unfolding of protein segments, rendering them insoluble (Hildebrandt et al., 2002).

### *VASCULAR DAMAGE*

Localised hyperthermia significantly alters blood flow through the vasculature surrounding tumours, resulting in endothelial swelling, increased plasma in the interstitium, microthrombosis and change in viscosity of blood cell membranes (Hildebrandt et al., 2002, Song, 1984, Vaupel et al., 1989). Tumour vasculature is chaotic, often resulting in poorly perfused and low pH areas that exhibit increased sensitivity to hyperthermia (Reinhold and Endrich, 1986). The morphological changes caused by hyperthermia in the surrounding vessels promote reduction in oxygen and nutrient supply, as well as acidosis within the tumour thus leading to increased apoptotic activity.

### *IMMUNE RESPONSE*

Hyperthermia is a natural response of the body to combat a wide range of infectious and immunologic attacks. The principle mediator of the immune effects seen post-hyperthermia treatment is due to the heat-induced expression of heat shock proteins (Rampersaud et al., 2010). HSPs are released by heat from both normal and cancerous cells, protecting cellular proteins by binding to exposed hydrophobic segments. As heat shock proteins released from dying cancerous cells contain tumour antigens, the immune system produces a surplus of CD8+ T-cells, which in turn have an anti-tumour response (Binder and Srivastava, 2005). Total body hyperthermia upregulates the production of natural killer cells. These cells act on natural killer activating ligands MICA (MHC-class-I-chain related gene A), expressed on tumour cells, which

leads to improved natural killer-mediated anti-tumour properties (Skitzki et al., 2009, Dayanc et al., 2008, Ostberg et al., 2007).

### *CHEMOHYPERTHERMIA TREATMENT*

When used in conjunction to chemotherapy, hyperthermia treatment enhances the cytotoxicity of many commonly used anti-neoplastic drugs, commonly termed thermal chemosensitization. There are several hypothesized reasons for the improved efficacy of neoplastic therapies when combined with heat. Firstly, due to the increased cellular membrane permeability and altered blood perfusion the chemotherapeutic agent more readily penetrates the tumour (Lammers et al., 2011). As discussed previously, hyperthermia therapy is directly cytotoxic, alters cell morphology and induces cell apoptosis. Finally, it has been shown that hyperthermia improves the cytotoxicity and consequent efficacy of mitomycin C, hypothesized to be the case for all chemotherapeutics (Paroni et al., 2001, Lammers et al., 2011).

A review of 22 studies aimed to assess the efficacy of chemohyperthermia (Lammers et al., 2011). The published data suggests a 59% reduction in recurrence of NMIBC and an improved bladder preservation profile in combined mitomycin C and hyperthermia therapy compared to mitomycin C alone. Local toxicity was reported to be higher in combined hyperthermia and mitomycin C therapy, however there was no major complications and patients appeared to self-heal (Colombo et al., 1996, Colombo et al., 2001). Hyperthermia (at 43°C) combined with mitomycin C, epirubicin, apaziquone (an analogue of mitomycin C) and gemcitabine have all shown synergism in decreasing cell proliferation in transitional cell carcinoma lines RT4, RT112, 253J and T24 (van der Heijden et al., 2005).

Apart from the above reported studies, there is limited published data on the efficacy, recurrence and progression of NMIBC treated with chemotherapy and hyperthermia treatment, and none that could be found involving gemcitabine. Further investigation into the cellular changes of combined chemotherapy and hyperthermia treatment may elucidate the benefits of this treatment for patients.

## **PATHOLOGICAL UROTHELIAL FUNCTIONING**

Extensive studies have focused on enhancing the toxicity of intravesical chemotherapies, with little regard to the effects of these treatments on the normal function of the bladder. As previously discussed, intravesical gemcitabine treatment for bladder cancer is associated with local side effects including chemical cystitis, dysuria and increased frequency and urgency of urination in a significant patient population. Little is known about the mechanism causing the reported urological side effects following intravesical treatment with this agent.

Changes in urothelial mediator release from the bladder have been reported in many pathological conditions of the bladder with symptoms similar to those reported by patients undergoing intravesical gemcitabine treatment. Significant increases in ATP release from the urothelium have been found in patients with painful bladder syndrome and with both idiopathic and neurogenic detrusor overactivity (Kumar et al., 2010, Kumar et al., 2007). In a feline interstitial cystitis model, the upregulated ATP release observed from the urothelium was partially mediated through a calcium-dependent vesicular exocytotic process (Birder et al., 2003). As the  $IP_3$  pathway mediates in part the intracellular calcium component, this suggests a putative role of the muscarinic receptors of the bladder in this pathological condition. Urine levels of prostaglandin  $E_2$  are increased in patients with overactive bladder syndrome (Kim et al., 2006). Urothelial prostaglandin  $E_2$  has also been suggested to modulate afferent nerve activity during urinary bladder inflammation via the EP1 receptor (Ikeda et al., 2006).

Interstitial cystitis is characterised by suprapubic pain and irritative voiding symptoms, which are similar to those reported by patients undergoing intravesical chemotherapy. The pathological process of interstitial cystitis is multifactorial, however it is often characterised by an inflammatory response and an increased release of mediators including histamines, substance P and cytokines (Theoharides et al., 2001, Sant, 2002).

No studies to date have investigated the effect of gemcitabine treatment alone or in combination with increased temperature on urothelial mediator release. A recent investigation of the first-line chemotherapy for NMIBC, mitomycin C, found enhanced release of the urothelial mediator prostaglandin  $E_2$  and the pro-inflammatory cytokine IL-8 after 24 hours recovery from treatment, with levels still elevated with 1- and 2-weeks of recovery (Kang et al., 2015). Furthermore,

doxorubicin treatment also induced release of the cytokines interleukin -8 and -1 $\beta$  24hours after treatment and enhanced stimulated (stretched) release of prostaglandin E<sub>2</sub> (Kang et al., 2013a).

## **CELL CULTURE AS A MODEL OF UROTHELIAL FUNCTION**

Urothelial, myofibroblast and detrusor muscle cells of the bladder have been shown to release mediators such as ATP with stretch (Cheng et al., 2011). Therefore, to assess urothelial cell specific effects, urothelial cell cultures were used as an *in vitro* model for investigating the effects of gemcitabine on the urothelium of the bladder. To investigate the cytotoxicity of gemcitabine on the normal cells of the bladder, malignant and non-malignant urothelial cells were treated with this drug. For comparative purposes, this protocol was repeated with mitomycin C, the first-line intravesical chemotherapy for superficial bladder cancer (Witjes and Hendricksen, 2008). The UROtsa cell line was used as the non-malignant urothelial cell line, with the RT4 and T24 malignant bladder cell lines used as models of superficial and invasive cancer respectively. Further descriptions of these cell lines will be outlined within the Materials and Methods section.

Mechanisms of gemcitabine cytotoxicity were examined between the malignant and non-malignant urothelial cell lines. The metabolism and mechanism of gemcitabine cytotoxicity has been reviewed within Chapter 1.0. Briefly, gemcitabine is up-taken into the cells via nucleoside transporters, with the human equilibrative nucleoside transporter (hENT1) having the greatest affinity for gemcitabine (Mackey et al., 1998). The intracellular effects of gemcitabine are multifactorial, including being incorporated into DNA and RNA, and the inhibition of ribonucleotide reductase and CTP-synthetase (Mini et al., 2006). The production of reactive oxygen species (ROS) has also been linked to the cytotoxicity of gemcitabine (Donadelli et al., 2011), and this was measured in this study.

RT4 cells have been shown to release ATP during basal conditions, enhanced with hypotonic simulation (McDermott et al., 2012, Mansfield and Hughes, 2014a, Kang et al., 2013a). These cells also release measureable levels of acetylcholine and prostaglandin E<sub>2</sub> during basal and stretched conditions (Kang et al., 2013a, McDermott et al., 2012). However, as these cells are



derived from malignant urothelial cells, the present study used the UROtsa cell line, cultured from normal human urothelial cells.

UROtsa cells have been shown to release ATP under basal and osmotic-stretch conditions, using isotonic and hypotonic solutions (Ochodnický et al., 2013, Mansfield and Hughes, 2014b). However, to the best of my knowledge, there have been no reports of basal and stimulated acetylcholine or prostaglandin E<sub>2</sub> release from UROtsa cells.

Rossi et al. (2001) proposed the UROtsa cell line as a useful cell culture model of human urothelium, reporting that these cells grow as an unstratified, undifferentiated monolayer with identical expression of methallothionin and heat shock gene expression to that of the basal cell layer of the urothelium. Furthermore, muscarinic receptor mRNA expression in the UROtsa cell line parallels expression in human urothelium (Tyagi et al., 2006), and it has recently been shown that UROtsa cells express functional purinergic receptors (Bakali et al., 2014). Additionally, mRNA for factors involved in the production or action of prostaglandins including COX-2, PTGES-1, EP2, EP4 and PGDH are expressed by UROtsa cells (Taylor et al., 2009).

The ability of UROtsa cells to imitate the release of mediators from the stretched urothelium was investigated in the present study, before examining the effect of gemcitabine treatment alone and in combination with hyperthermia on these mediators as a model to investigate the urothelial cell-specific effects.

## **3.2 AIMS**

The aim of the present study was to investigate the cytotoxicity of gemcitabine and its effects on urothelial function. The specific aims of this experiment were separated into three arms:

### **AIM 1: INVESTIGATION OF THE CYTOTOXICITY OF GEMCITABINE**

- To determine the potency of gemcitabine on non-malignant vs malignant urothelial cells and compare to the current standard chemotherapeutic mitomycin
- To investigate the effect of combined gemcitabine or mitomycin C therapy with hyperthermia treatment on cytotoxicity

### **AIM 2: INVESTIGATION OF THE MECHANISMS OF GEMCITABINE TOXICITY**

- To determine if reactive oxygen species production is altered in non-malignant vs malignant urothelial cells after gemcitabine treatment
- To assess the contribution of the human equilibrative nucleoside transporter 1 (hENT1) in gemcitabine toxicity

### **AIM 3: INVESTIGATION OF THE MECHANISMS OF ADVERSE EFFECTS AFTER IN VITRO GEMCITABINE TREATMENT**

- To examine the ability of UROtsa cells to release the urothelial mediators ATP, acetylcholine and prostaglandin  $E_2$  under basal and stretched conditions
- To assess the immediate and late effects of gemcitabine treatment alone and in combination with hyperthermia on the release of the aforementioned mediators
- To determine the effects of gemcitabine treatment alone and in combination with hyperthermia on inflammatory cytokine release

### **3.3 MATERIALS AND METHODS**

#### **CELL CULTURE**

Three cell lines were used within this study. The bladder cancer cell lines RT4 and T24 were obtained from the European Collection of Cell Cultures, while the non-malignant human urothelial cell line UROtsa was received as a gift from Professor Scott Garrett (University of North Dakota). All cells were stored in freeze medium containing DMSO (10%) in liquid nitrogen until required.

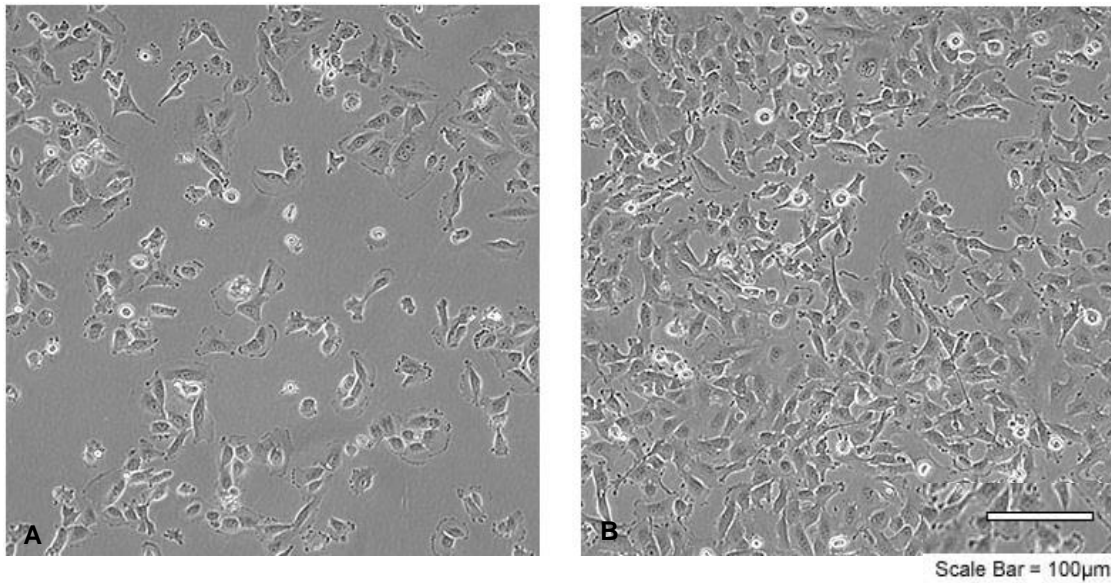
#### **RT4 CELLS**

RT4 cells are derived from a recurring transitional cell papilloma tumour of the human urinary bladder and are widely used as a model of superficial bladder cancer. Culturing of these cells produces consistent morphology and chromosome patterns, and xenografts of RT4 cells into both mouse and hamster specimens have been shown to produce tumours of similar morphology to those of original biopsies (Masters et al., 1986, Rigby and Franks, 1970).

RT4 cells were cultured in McCoy's 5A culture medium (Invitrogen, Victoria, Australia) containing L-glutamine and phenol red, supplemented with 10% foetal bovine serum (FBS), and 500 U/mL penicillin-streptomycin (Invitrogen, Victoria, Australia), as described previously (McDermott et al., 2012). Cells were maintained at 37°C in a 5% CO<sub>2</sub> incubator, passaged when 80-90% confluent, with a doubling time of approximately 35 hours (Dubeau and Jones, 1987). The subculture method is described later in this section.

## T24 CELLS

T24 cells are a derivative of a human urinary bladder transitional cell carcinoma as shown in **Figure 3.1** and exhibit characteristics of high grade, invasive tumours, including spreading and separating during cell motility (Kabaso et al., 2011). T24 cell growth in tissue culture has been characterised by a multi-layered, mixed epithelial to fibroblastoid morphology, and the presence of tumour specific antigens (TSA) (Bubenik et al., 1973). This cell line was grown in the same culture medium used for RT4 cells, described previously.



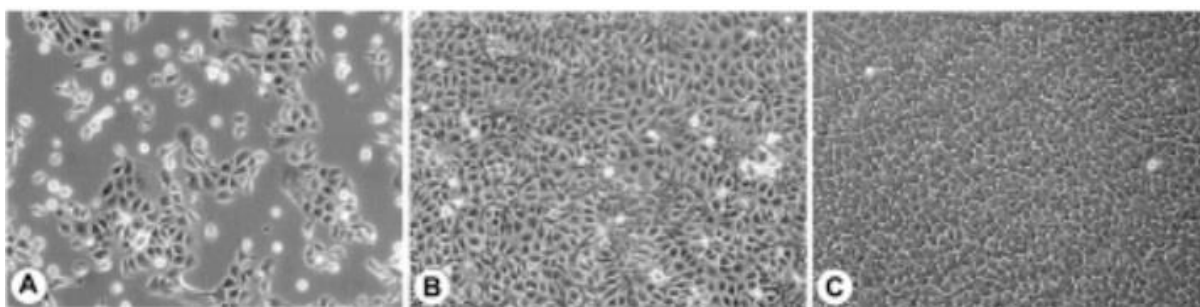
**Figure 3.1:** T24 cells at A) approximate 50% confluence and B) approximate 90% confluence. Pictures from the American Type Culture Collection database.

## UROtsa CELLS

The UROtsa cell line is an immortalized cell line from cultures of the normal human urothelium from the proximal ureter using the SV40 T-antigen (Petzoldt et al., 1995). These cells are considered non-tumorigenic due to their inability to form colonies in soft agar and nude mice, growing as a contact-inhibited monolayer in an undifferentiated form consistent with the basal cells of the human urothelium, as seen in **Figure 3.2** (Somji et al., 2008, Petzoldt et al., 1995).

UROtsa cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM - Sigma-Aldrich, NSW, Australia) supplemented with 5% FBS, 1% glucose and 1% penicillin-streptomycin (Invitrogen, Victoria, Australia). For experiments, UROtsa cells were used and grown in serum-supplemented and serum-free media, for models of proliferative and non-proliferative urothelial cells respectively. Incubation of UROtsa cells in serum-free media leave the cells in a non-proliferative state while UROtsas grown in serum-supplemented media are used as a model of undifferentiated, proliferative urothelial cells that grow as a single cell monolayer (Rossi et al., 2001). In the human bladder, the normal urothelium is mitotically quiescent with a slow rate of cell turnover and a low proportion of cells in the cell cycle (Marceau, 1990). Therefore, to best mimic this condition, consequent experiments examining urothelial functioning used UROtsas in the non-proliferative state.

However, in response to tissue damage, the urothelium has a high capacity for proliferation (Limas, 1993). It has been shown that growth of UROtsa cells in serum-free medium consisting of a mixture of DMEM and Ham's F12, supplemented with selenium, insulin, transferrin, hydrocortisone, triiodothyronine and epidermal growth factor results in the differentiation of these cells to form a multilayered, stratified cell cultures. These cells display characteristics found in differentiated intermediate urothelial cells, including desmosomes and gap junctions (Rossi et al., 2001, Lewis, 2000, Jost et al., 1989). Therefore, UROtsa cells are an excellent model of the human urothelial cells, as they are non-proliferative in unsupplemented media, but have the capacity to rapidly differentiate.



**Figure 3.2: UROtsa cell culture at stages of confluency (10X magnification). A) UROtsas at approximately 50% confluency 1 day after subculture, B) confluent UROtsa cells maintained in serum-containing media 7-days after subculture, C) UROtsa cells 14-days after subculture remaining as a monolayer (Rossi et al., 2001)**

## CELL SUBCULTURE

The subculture procedure was standard across all cell lines, with the exception of culture media used. When cells were 80-90% confluent, media was removed and cells were washed with approximately 6mL of 1X phosphate buffered solution (PBS, Life-Technologies, Victoria, Australia). Two mL of trypsin-EDTA (Life-Technologies, Victoria, Australia) was added to the cells and cultures incubated at 37°C in a 5% CO<sub>2</sub> incubator until the cells detached. Six mL of the respective culture media for the cell line undergoing passage was added and the resulting cell suspension was transferred to a 15mL centrifuge tube and cells were spun in a centrifuge at 800rpm for 3 minutes. The supernatant solution was discarded and cells were resuspended in 6mL of fresh culture media for passaging. Typical cell splits were 1:3 for UROtsa and RT4 cells, and 1:4 for T24 cells. Cell viability was assessed by trypan blue exclusion using Countess Automated Cell Counter (C10227 Invitrogen, Victoria, Australia) and only cultures with viability >95% were used for experiments. A final volume of 20mL was added to the T75 flasks after passage.

## AIM 1: INVESTIGATION OF CYTOTOXICITY OF GEMCITABINE

### CELL SEEDING DENSITIES

Cells were seeded in 24 or 96-well microtiter plates at a density determined during initial optimisation experiments. **Table 3.1** describes the cell densities plated for the various experiments. Cells were left to attach for 24 hours in their respective media before treatment with gemcitabine or mitomycin C and consequent experiments.

**Table 3.1: Typical seeding densities used for experiments for each cell line, NA – not applicable**

Cell Line	Seeding Density in 96 well plates	Seeding density in 24 well plates	Seeding Density in T25 flasks
<b>Non-Proliferative UROtsa</b>	$2.5 \times 10^4$ per well	$1.5 \times 10^5$ per well	$2 \times 10^6$ per flask
<b>Proliferative UROtsa</b>	$1.0 \times 10^4$ per well	NA	NA
<b>RT4</b>	$1.0 \times 10^4$ per well	NA	NA
<b>T24</b>	$0.5 \times 10^4$ per well	NA	NA

### GEMCITABINE TREATMENT

Cells were treated with increasing concentrations of gemcitabine from 4ng/mL (15nM) up to the maximum clinical dose of 40mg/mL (150mM). After incubation at 37°C or 42°C for 60 minutes (clinical duration of treatment), cells were rinsed twice with sterile PBS before aspiration and measurement of viability using a resazurin reduction assay.

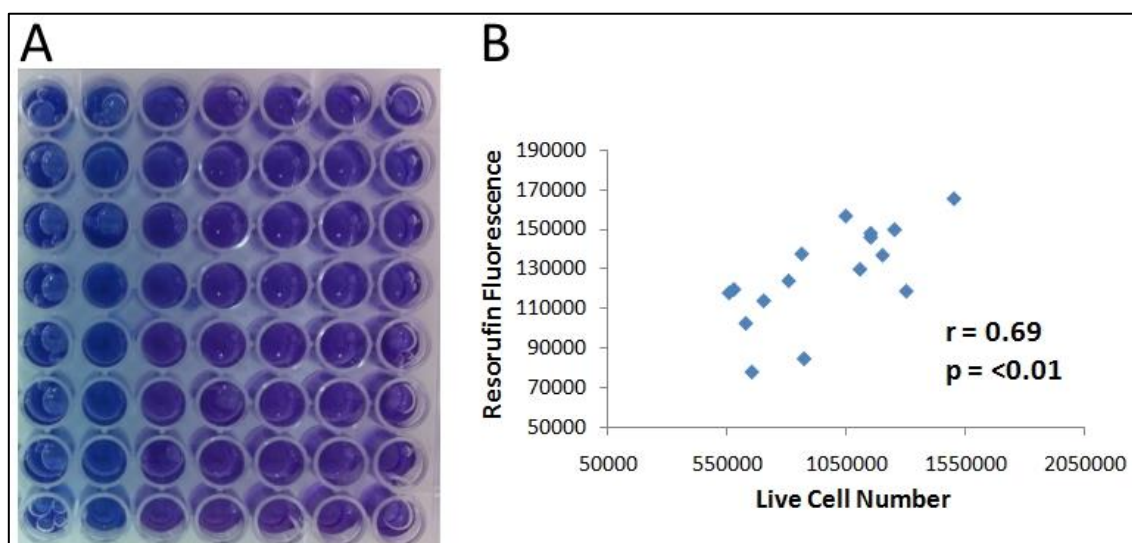
### MITOMYCIN C TREATMENT

Cells were treated with increasing concentrations of mitomycin C from 20ng/mL (60nM) up to the maximum clinical dose of 2mg/mL (6mM). After incubation at 37°C or 42°C for 120 minutes (clinical duration of treatment), cells were rinsed twice with sterile PBS before aspiration and measurement of viability using a resazurin reduction assay.

### RESAZURIN VIABILITY ASSAY

The effects of the treatments were measured using the resazurin reduction assay, a commonly used assay for cell proliferation, viability and cytotoxicity without causing damage to the cells (O'Brien et al., 2000). Blue resazurin is reduced intracellularly by viable, metabolically active cells to resorufin and dihydroresorufin. Resorufin is pink and highly fluorescent, with fluorescence increasing proportionally to the number of cells present. A significant positive correlation between live cell number and resorufin fluorescence was determined, as shown in **Figure 3.3** ( $p < 0.01$ ).

After treatment and washing, 100 $\mu$ L of 44 $\mu$ M resazurin solution made up in the respective culture media was incubated at 37°C for approximately 2 hours or until control wells had developed in colour. This process was repeated 24, 48 and 72 hours after treatment, replacing the removed resorufin product with 200 $\mu$ L of the respective culture media. Fluorescence of the reduced resorufin product was then measured using a Modulus Microplate reader (Ex. 530/ Em. 590nm). All experiments were performed in triplicate and repeated at least six times.



**Figure 3.3:** Resazurin in a 96 well plate. (A) Cell number increasing from left to right, colour indicative of resorufin from reduced resazurin and (B) Pearson correlation analysis of the relationship between live cell number and corresponding resorufin



## **AIM 2: MECHANISM OF GEMCITABINE TOXICITY**

### **GEMCITABINE AND NBMPR TREATMENT**

Cell viability was also assessed after treatment of urothelial cells with gemcitabine in the presence of 1 $\mu$ M NBMPR (Tocris, NSW, Australia), an inhibitor of the hENT1 equilibrative nucleoside transporters. Cells were seeded in 96-well plates and left to attach for 24 hours as previously described. Cells were incubated with gemcitabine and 1 $\mu$ M NBMPR at 37°C for 60 minutes, rinsed twice with sterile PBS before aspiration and replacement with culture medium and incubated for up to 72 hours. Measurement of viability used a resazurin reduction assay as described previously.

### **REACTIVE OXYGEN SPECIES PRODUCTION**

The production of reactive oxygen species (ROS) was measured using the 2', 7'-dichlorofluorescein diacetate assay (DCFH-DA) (Sigma-Aldrich, Australia). This assay is based on the intracellular oxidation and consequent de-esterification of the non-fluorescent DCFH-DA to the highly fluorescent 2', 7'-dichlorofluorescein (DCF). The cell lines were seeded in 96-well microtiter plates as previously described. Cells were treated with increasing concentrations of gemcitabine at 37°C for 1 hour, before being washed with PBS and incubated for a further 24, 48 or 72 hours in their respective culture media. Cells were incubated with 10 $\mu$ M DCFH-DA solution, protected from light for 40 minutes. The production of ROS was quantified using a Modulus Microplate reader (Ex. 530/ Em. 590nm). DCF fluorescence was normalized to controls using corresponding resazurin reduction data.

### AIM 3: MECHANISMS OF ADVERSE EFFECTS AFTER INTRAVESICAL GEMCITABINE

#### TREATMENT

##### TIME COURSE MEDIATOR RELEASE: OPTIMISATION EXPERIMENTS

UROtsa cells cultured in serum-free media were used for all urothelial mediator release studies. Twenty-four well plates were seeded at a density of  $1.2 \times 10^5$  cells per well and incubated in serum-free media overnight. To determine the optimal incubation time of mediator release from UROtsa cells with isotonic and hypotonic solutions, three time points (5, 10 and 15 minutes) were tested and samples analysed.

Cells were washed twice with PBS and replaced with 200 $\mu$ L isotonic solution (**Table 3.2**) for 15 minutes. This solution was replaced with 150 $\mu$ L isotonic solution and incubated for 5, 10 or 15 minutes at 37°C, before collection for basal mediator release. For stretch release of urothelial cell mediators, 150 $\mu$ L hypotonic solution (**Table 3.2**) was added to the wells and incubated for 5, 10 or 15 minutes before being collected for analysis.

**Table 3.2: Ingredients used for isotonic and hypotonic solutions. All chemicals obtained from Sigma Australia**

Isotonic Solution	Hypotonic Solution
NaCl 130mM	KCl 5mM
KCl 5mM	CaCl <sub>2</sub> 1.5mM
CaCl <sub>2</sub> 1.5mM	MgCl <sub>2</sub> 1mM
MgCl <sub>2</sub> 1mM	NaHEPES 25mM
NaHEPES 25mM	BSA (0.1%) 1mg/mL
BSA (0.1%) 1mg/mL	Glucose 5mM
Glucose 5mM	

Mediators were measured using commercially available ATP, acetylcholine and prostaglandin E<sub>2</sub> kits as described in Section 2.0.

UROtsa cells were found to release ATP, acetylcholine and prostaglandin E<sub>2</sub> both under basal and hypotonic-stimulated conditions (**Figure 3.4**). Stimulated release of ATP was significantly higher ( $p<0.001$ ) than basal release at all time-points tested, decreasing in a time dependent manner (**Figure 3.4A**). Urothelial cells released acetylcholine at both basal and stimulated conditions (**Figure 3.4B**); however there was a significant increase with stretch only in the 10 ( $p<0.01$ ) and 15 minute ( $p<0.05$ ) incubation groups. Prostaglandin E<sub>2</sub> was released from UROtsa cells basally and with stretch, significantly after incubation in hypotonic solution for 5, 10 ( $p<0.001$ ) and 15 minutes ( $p<0.01$ ). The optimum time for release was determined to be after a 10 minute incubation period, which yielded significant increased stimulated release from basal of the urothelial mediators ATP, acetylcholine and prostaglandin E<sub>2</sub>.

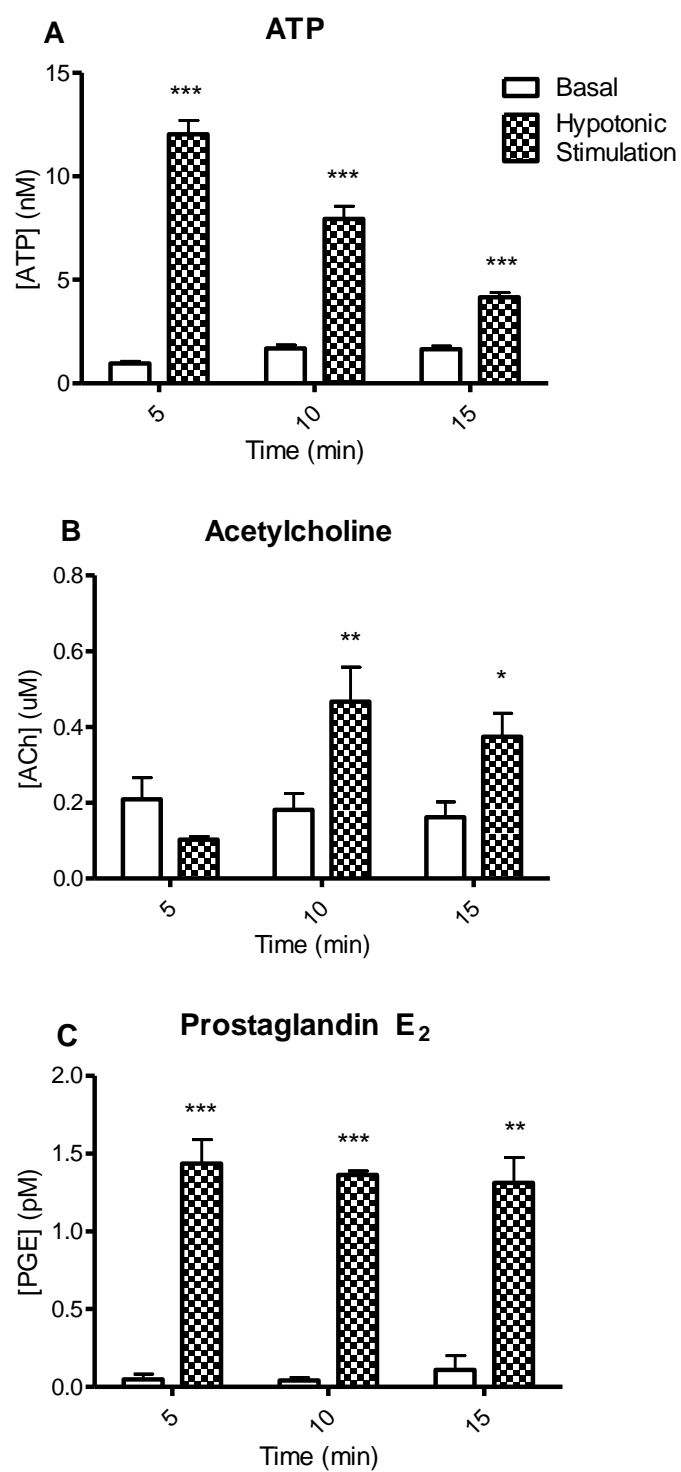


Figure 3.4: Basal and hypo-osmotically stimulated ATP (A), acetylcholine (B) and prostaglandin E<sub>2</sub> (C) mediator release from UROtsa cells after 5, 10 and 15 incubation period. Data shown as the mean  $\pm$  SEM ( $n \geq 3$ ), analysed by a paired two-tailed t-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs basal release).

#### UROTHELIAL MEDIATOR RELEASE: IMMEDIATE EFFECT OF GEMCITABINE ALONE AND IN COMBINATION WITH HYPERTHERMIA

UROtsa cells were plated as previously described and incubated in unsupplemented media overnight. Cells were treated with gemcitabine diluted in serum-free media (0 – 4mg/mL) and incubated at 37°C or 42°C for 1 hour. Incubation media was aspirated and cells were washed twice with sterile PBS prior to the addition of 200µL isotonic solution, before removal and replacement with 150µL of fresh isotonic solution, incubated for a 10 minute period. Samples of urothelial mediator release were obtained as described above, stored frozen at -30°C before measurement using commercially available ATP, acetylcholine and prostaglandin E<sub>2</sub> kits as described in Section 2.0.

#### UROTHELIAL MEDIATOR RELEASE: LATE (24H) EFFECT OF GEMCITABINE ALONE AND IN COMBINATION WITH HYPERTHERMIA

UROtsa cells were plated as previously described and incubated in unsupplemented media overnight. Cells were treated with gemcitabine diluted in serum-free media (0 – 4mg/mL) and incubated at 37°C or 42°C for 1 hour. Incubation media was aspirated and cells were washed twice with sterile PBS before the addition of serum-free culture media. Cells were incubated for a further 24 hours. Prior to the addition of 200µL isotonic solution, cells were rinsed twice with sterile PBS solution. Samples of urothelial mediator release were obtained as described above, stored frozen at -30°C before measurement using commercially available ATP, acetylcholine and prostaglandin E<sub>2</sub> kits as described in Section 2.0.

#### INFLAMMATORY CYTOKINE ANALYSIS

UROtsa cells were seeded into T25 flasks at densities according to **Table 1**, grown to confluence, and subsequently incubated in serum-free culture media for a further 24 hours, before treatment with 4mg/mL gemcitabine incubated at 37°C or 42°C for 1 hour. Cells were washed twice with sterile PBS and incubated for a further 24 hours in serum-free media. This cell free media was collected and the presence of inflammatory cytokines (Interleukin-1β, -6, -8,

-10, 12p70 and tumour necrosis factor) was analysed using a BD Cytometric Bead Array Human Inflammatory Cytokines Kit according to the manufacturer's protocol. This kit uses beads conjugated with a specific antibody to each of the cytokines tested. A detection agent comprised of a mixture of phycoerythrin-conjugated antibodies provides a fluorescent signal in proportion to the amount of bound analyte. When the capture beads and detector reagent are incubated with an unknown sample containing recognized analytes, a sandwich complex is formed (capture bead + analyte + detection reagent). These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

Standard and sample fluorescence were analysed on a BD FACS Calibur flow cytometer. The concentration of inflammatory cytokines measured were normalised to controls using corresponding resazurin reduction data.

## 3.4 RESULTS

### PART A – CYTOTOXICITY OF GEMCITABINE

#### CYTOTOXICITY OF GEMCITABINE WITH COMPARISON TO MITOMYCIN C

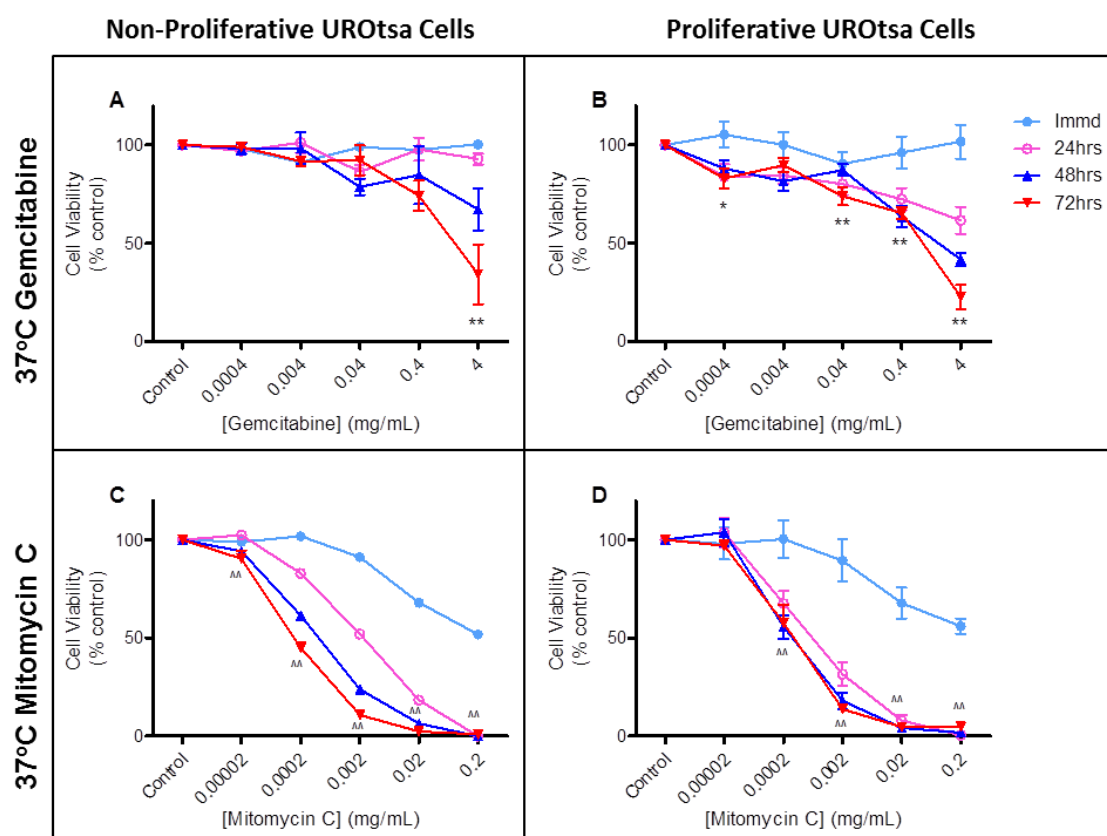
The cytotoxicity of gemcitabine with comparison to mitomycin C was assessed in malignant and non-malignant urothelial cells, measured in terms of changes in cell viability immediately, 24, 48 and 72 hours post treatment. Both gemcitabine and mitomycin C reduced viability of all cell lines in a time and concentration dependent manner (**Figure 3.5 & 3.6**). The clinical concentrations of 40mg/mL gemcitabine and 2mg/mL mitomycin C were initially tested on all cells lines. Cell viability was reduced to <1% across all cell lines for both drugs (data not shown), and these concentrations were consequently not tested for subsequent experiments.

The concentration of gemcitabine or mitomycin C which resulted in a 50% reduction in cell viability ( $LC_{50}$ ) was used to determine the relative potency of these drugs in each of the cell lines. A significant time dependent decrease ( $p<0.001$ ) in the  $LC_{50}$  was observed across all cell lines treated with gemcitabine or mitomycin C, with the exception of the non-proliferative UROtsa cells ( $p=0.09$ , **Figure 3.5A, Table 3.3 & 3.4**).

The effects of gemcitabine on cell viability were significantly different between the cell lines measured 72 hours post treatment ( $p>0.001$ ) (**Figure 3.7A, Table 3.5**). Cytotoxicity of gemcitabine was greatest in the urothelial cancer cell lines RT4 and T24, with an approximate 11,000 and 25,000-fold greater potency respectively with comparison to the non-malignant UROtsa cell line in its non-proliferative state (**Table 3.5**).

Mitomycin C was 10-fold more potent on the UROtsa cell lines (in both non-proliferative and proliferative states) with comparison to the malignant bladder cell lines RT4 and T24 (**Figure 3.7B, Table 3.5**). In the non-malignant UROtsa cells, mitomycin C was significantly more cytotoxic than gemcitabine. The  $LC_{50}$  values for mitomycin C were significantly ( $p<0.001$ ) lower with comparison to those for gemcitabine in both the non-proliferative [ $0.5(0.4-0.5)\mu\text{M}$  vs  $11.5(4.5-28.9)\text{mM}$  for gemcitabine] and proliferative [ $0.9(0.6-1.2)\mu\text{M}$  vs  $2.3(1.2-4.4)\text{mM}$  for gemcitabine] forms of UROtsa measured 72 hours post treatment (**Table 3.5**).

In comparison, gemcitabine was significantly more toxic than mitomycin C in the urothelial cancer cell lines RT4 and T24. The mean LC<sub>50</sub> values in these cell lines were significantly lower for gemcitabine compared to mitomycin C, 1.0(0.5-2.0)  $\mu$ M compared to 18.2(11.2-29.6)  $\mu$ M ( $p < 0.001$ ) in RT4 cells and 0.4(0.3-0.6)  $\mu$ M compared to 5.1(3.6-7.3)  $\mu$ M ( $p < 0.001$ ) in T24 cells (Table 5).



**Figure 3.5: Effect of 1 hour gemcitabine treatment (A & B) or 2 hour mitomycin C treatment incubated at 37°C on UROtsa cell viability in their non-proliferative and proliferative states, assessed immediately, 24, 48 and 72 hours post treatment. Data are shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ), with the 72hr group analysed by a one-way ANOVA with Dunnett post-test (\*  $p > 0.05$ , \*\*  $p > 0.01$  vs gemcitabine control, ^^  $p > 0.01$  vs mitomycin C control).**



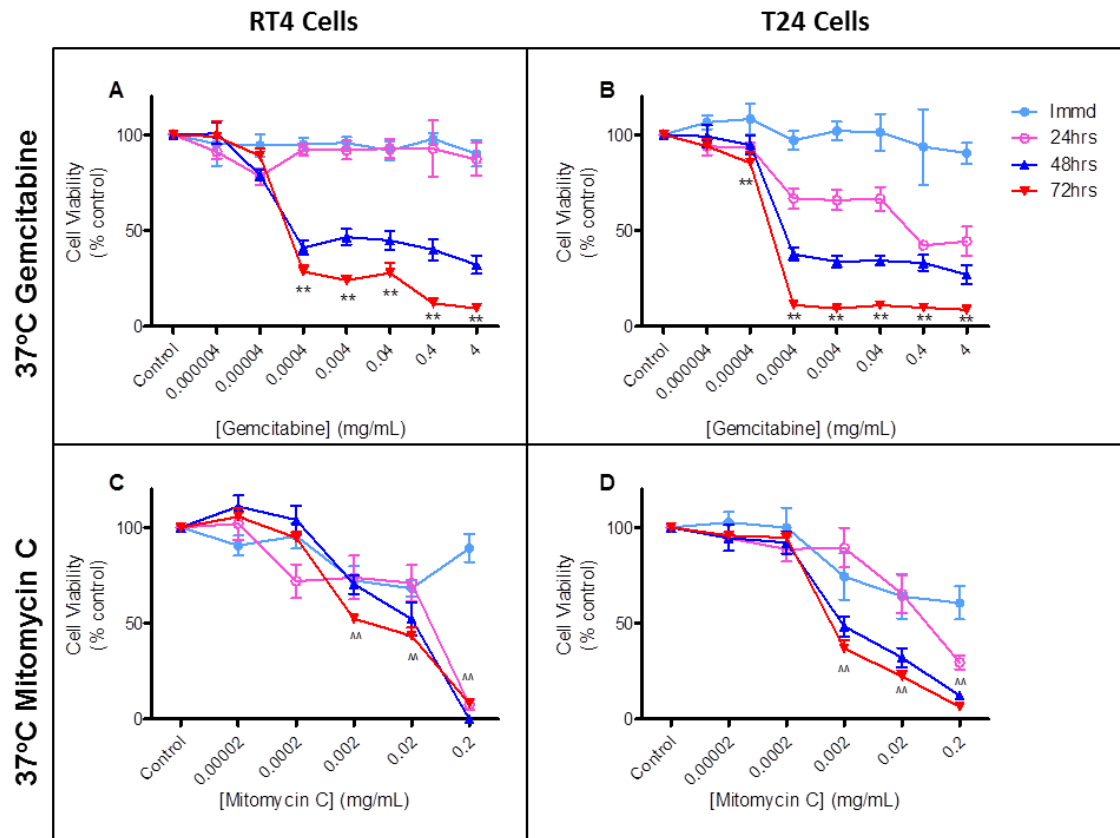


Figure 3.6: Effect of 1 hour gemcitabine treatment (A & B) or 2 hour mitomycin C treatment incubated at 37°C on RT4 and T24 cell viability assessed immediately, 24, 48 and 72 hours post treatment. Data are shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ), with the 72hr group analysed by a one-way ANOVA with Dunnett post-test (\*\*  $p > 0.01$  vs gemcitabine control, ^^  $p > 0.01$  vs mitomycin C control).

**Table 3.3: Cytotoxic effect of gemcitabine and mitomycin C on UROtsa cells in their non-proliferative and proliferative states, measured immediately, 24, 48 and 72 hours after treatment at 37°C using resazurin reduction assay. Data is reported as pLC50 and standard error (n=6), analysed with an F-test (multiple comparison of the non-linear curve fits).**

37°C Incubation												
Non-Proliferative UROtsa Cells							Proliferative UROtsa Cells					
Gemcitabine Treatment			Mitomycin C Treatment				Gemcitabine Treatment			Mitomycin C Treatment		
pLC50	Std. Error	p value	pLC50	Std. Error	p value		pLC50	Std. Error	p value	pLC50	Std. Error	p value
24hrs	-1.2	0.9		-2.7	0.03		-0.2	1.0		-3.2	0.1	
48hrs	-1.3	0.5	p=0.09	-3.4	0.02	p<0.001	-2.1	0.2	p<0.001	-3.5	0.1	p<0.001
72hrs	-2.0	0.2		-3.8	0.03		-2.6	0.1		-3.5	0.1	

**Table 3.4: Cytotoxic effect of gemcitabine and mitomycin C on RT4 and T24 cells, measured immediately, 24, 48 and 72 hours after treatment at 37°C using resazurin reduction assay. Data is reported as pLC50 and standard error (n=6), time dependent cell death analysed with an F-test (multiple comparison of the non-linear curve fits). Ambiguous regression data was excluded from analysis.**

37°C Incubation												
RT4 Cells							T24 Cells					
Gemcitabine Treatment			Mitomycin C Treatment				Gemcitabine Treatment			Mitomycin C Treatment		
pLC50	Std. Error	p value	pLC50	Std. Error	p value		pLC50	Std. Error	p value	pLC50	Std. Error	p value
24hrs	Excluded		-1.7	0.2			-2.9	0.4		-1.3	0.2	
48hrs	-4.4	0.3	p<0.001	-1.8	0.1	p<0.001	-5.3	0.2	p<0.001	-2.4	0.1	p<0.001
72hrs	-6.0	0.1		-2.2	0.1		-6.4	0.1		-2.8	0.1	

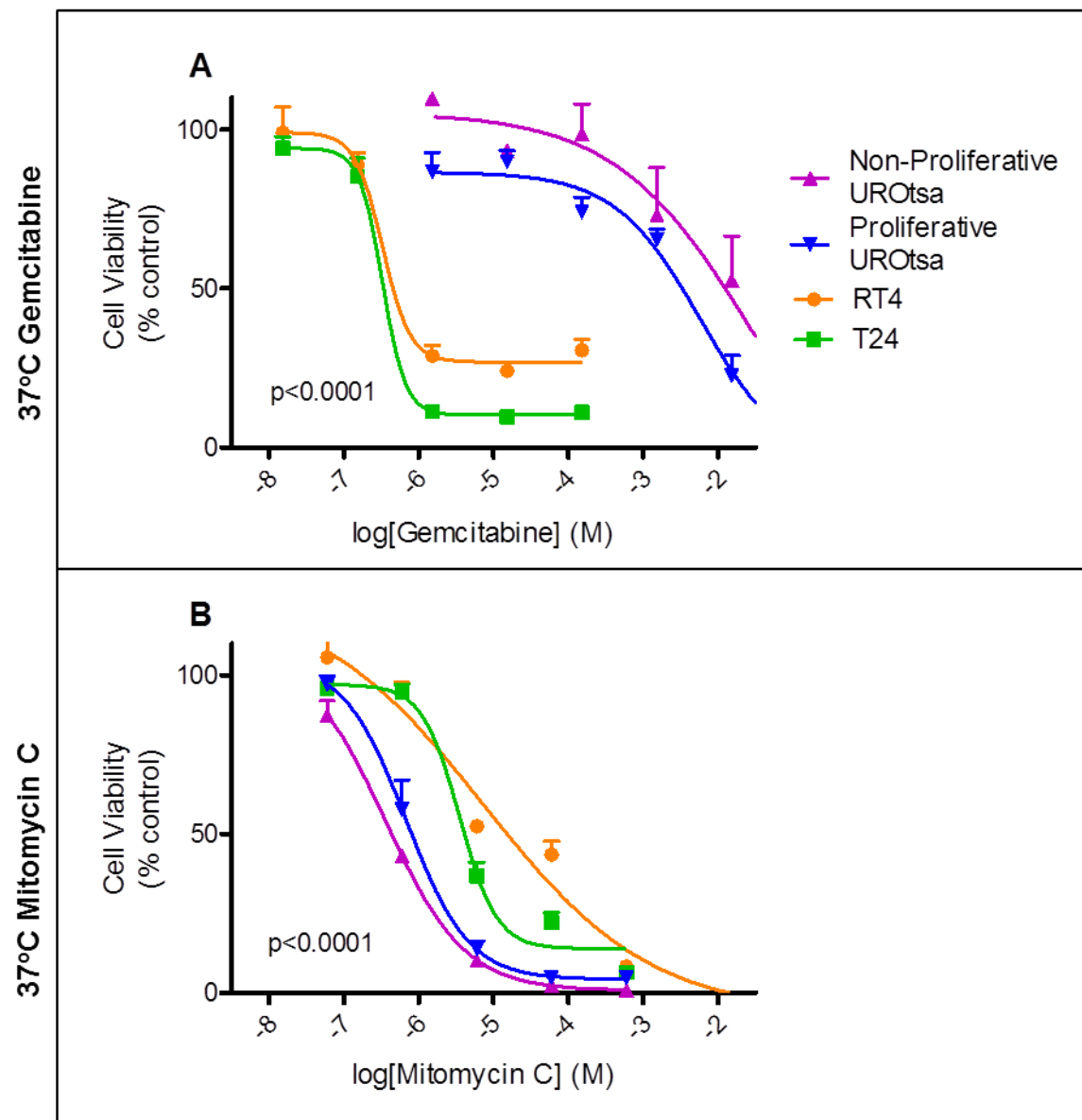


Figure 3.7: Effect of gemcitabine (A) and mitomycin C (B) on urothelial cell viability was measured 72hours after pre-treatment. Data are shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ), analysed with an F-test (multiple comparison of the non-linear curve fits).

**Table 3.5: LC50 of urothelial cell lines 72 hours post gemcitabine and mitomycin C treatment. LC<sub>50</sub>s were calculated as mean values of n≥6, analysed with an F-test (multiple comparison of the non-linear curve fits).**

LC50 72hours treatment	at post	Non- Proliferative UROtsa	Proliferative UROtsa	RT4	T24	Comparison of drug cytotoxicity between cell lines
<i>Gemcitabine</i> 37°C (95% CI)		11.5 (4.5- 28.9) mM	2.3 (1.2-4.4) mM	1.0 (0.5-2.0) µM	0.4 (0.3-0.6) µM	P<0.001
<i>Mitomycin</i> 37°C (95% CI)	C	0.5 (0.4-0.5) µM	0.9 (0.6-1.2) µM	18.2 (11.2- 29.6) µM	5.1 (3.6-7.3) µM	P<0.001
<b>Comparison of cytotoxicity of gemcitabine to mitomycin C on cell lines</b>		P<0.001	P<0.001	P<0.001	P<0.001	

## CYTOTOXICITY OF GEMCITABINE WITH COMPARISON TO MITOMYCIN C: EFFECTS OF TEMPERATURE

The effect of increased temperature in conjunction with gemcitabine or mitomycin C treatment was assessed in malignant and non-malignant urothelial cells, measured immediately, 24, 48 and 72 hours after pre-treatment at 42°C. Similar to treatment at 37°C, both gemcitabine and mitomycin C treatment at 42°C reduced viability of all cell lines in a time and concentration dependent manner (**Figure 3.8 & 3.9**).

A significant time dependent decrease ( $p \leq 0.001$ ) in the  $LC_{50}$  was observed across all cell lines treated with gemcitabine or mitomycin C at 42°C (**Table 3.6 & 3.7**).

Combined hyperthermia and gemcitabine treatment had a significantly greater cytotoxic effect on UROtsa cells in the non-proliferative state with comparison to gemcitabine treatment alone, with a significant decrease in  $LC_{50}$  [1.2(0.3-4.9)mM to 11.5(4.5-28.9)mM respectively,  $p=0.01$ , **Table 3.5 & 3.8**]. This effect was not observed in the other cell lines tested. The cytotoxicity of gemcitabine combined with hyperthermia had similar selectivity for malignant urothelial cells as when measured at 37°C by a factor greater than 1,000 to the malignant RT4 and T24 cells with comparison to the non-malignant UROtsa cells ( $p < 0.001$ , **Table 3.8, Figure 3.10A**).

Similarly, comparison of  $LC_{50}$  values indicated that combined hyperthermia and mitomycin C had a significant increased cytotoxic effect on UROtsa cells in the non-proliferative state [0.1(0.1-0.1)  $\mu$ M to 0.5(0.4-0.5) $\mu$ M,  $p < 0.001$ ] and malignant RT4 cells with comparison to mitomycin C treatment alone [0.8(0.6-1.0)  $\mu$ M to 18.2(11.2-29.6)  $\mu$ M,  $p < 0.001$ ]. The cytotoxicity in T24 cells was not significantly affected ( $p > 0.05$ ) by this combined regime.

The cytotoxicity of mitomycin C in combination with hyperthermia was again significantly different ( $p > 0.001$ ) between the urothelial cell lines tested, with UROtsa cells more susceptible to the cytotoxicity of treatment than the malignant cell lines (**Table 3.8, Figure 3.10B**).

Despite the additive cell killing effect of gemcitabine in combination with hyperthermia on UROtsa cells, mitomycin c was significantly more toxic than gemcitabine on both the non-

proliferative ( $p<0.001$ ) and proliferative ( $p<0.001$ ) UROtsa cells when pre-incubated at 42°C (Table 3.8).

In comparison, there was no difference between the cytotoxicity of gemcitabine or mitomycin C on RT4 and T24 cells when pre-incubated at 42°C (Table 8).

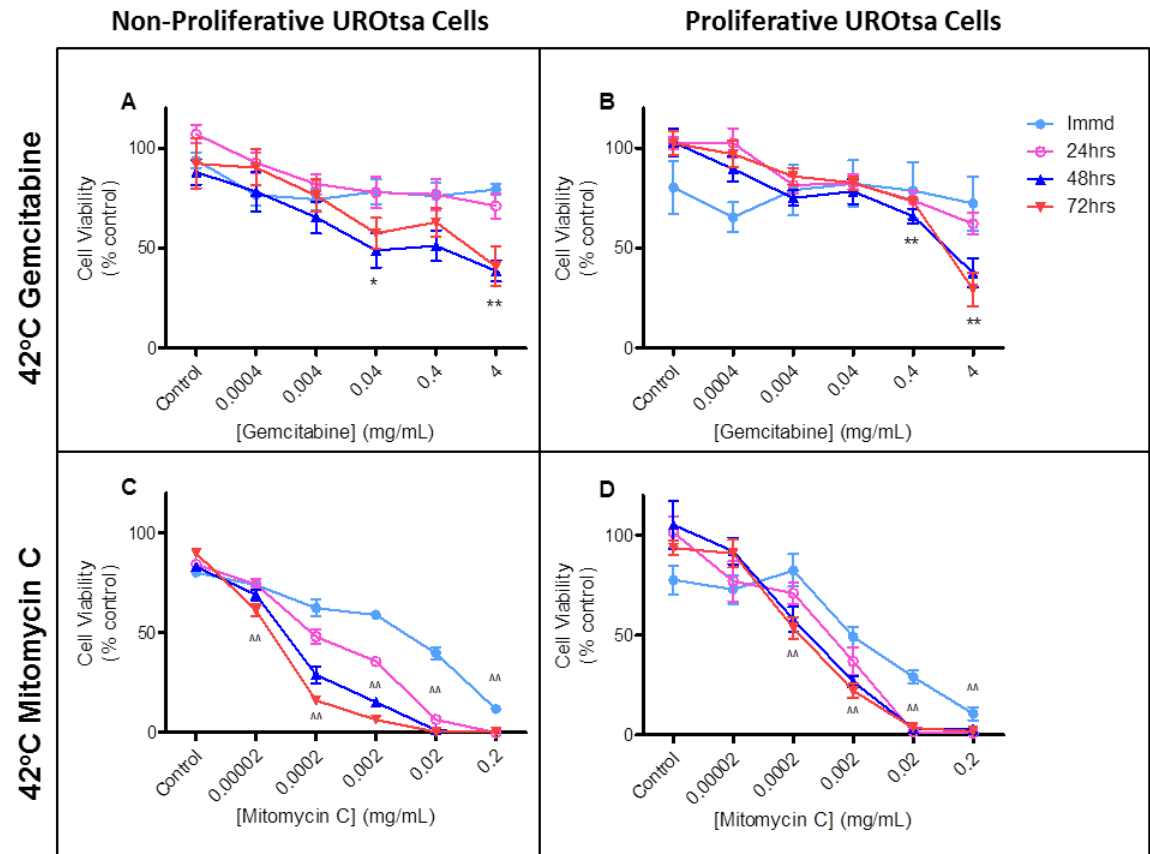


Figure 3.8: Effect of 1 hour gemcitabine treatment (A & B) or 2 hour mitomycin C treatment incubated at 42°C on UROtsa cell viability in their non-proliferative and proliferative states, assessed immediately, 24, 48 and 72 hours post treatment. Data are shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ), with the 72hr group analysed by a one-way ANOVA with Dunnett post-test (\*  $p>0.05$ , \*\*  $p>0.01$  vs gemcitabine control, ^^  $p>0.01$  vs mitomycin C control).

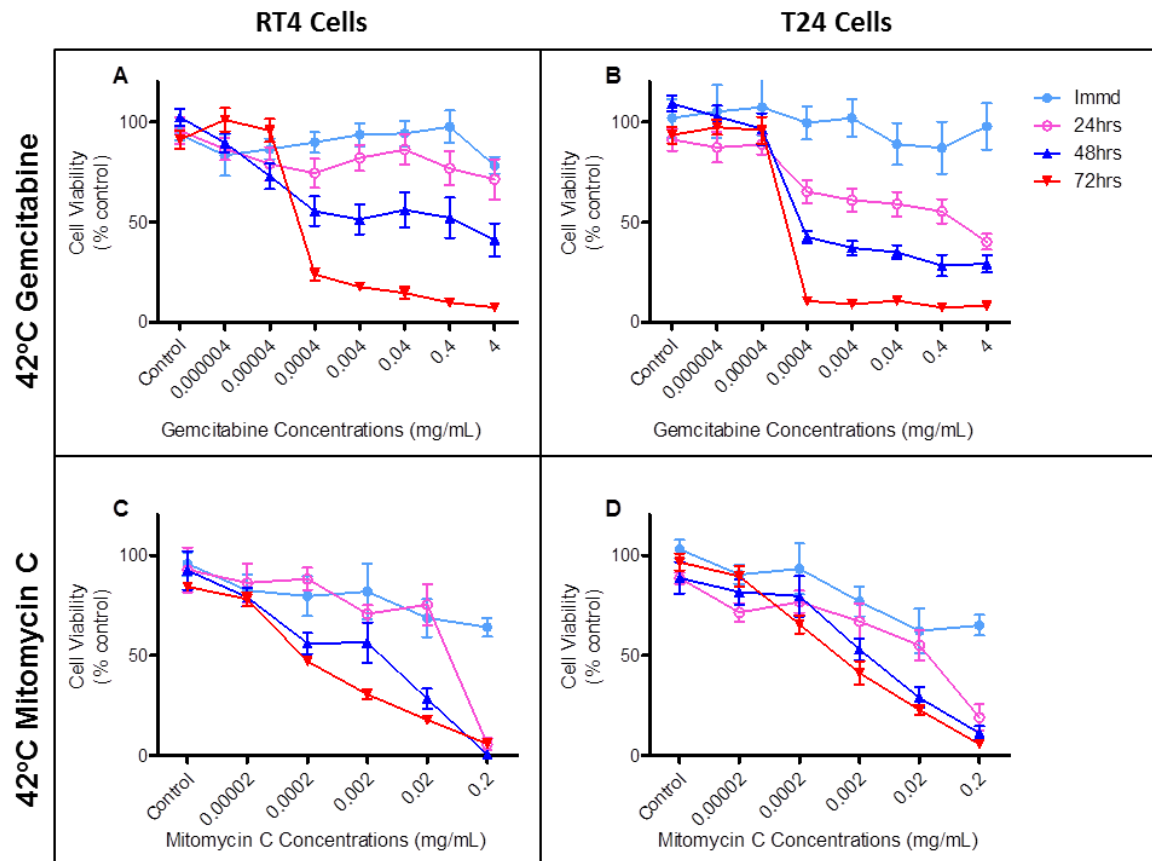


Figure 3.9: Effect of 1 hour gemcitabine treatment (A & B) or 2 hour mitomycin C treatment incubated at 37°C on RT4 and T24 cell viability assessed immediately, 24, 48 and 72 hours post treatment. Data are shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ), with the 72hr group analysed by a one-way ANOVA with Dunnett post-test (\*  $p > 0.05$ , \*\*  $p > 0.01$  vs gemcitabine control, ^^  $p > 0.01$  vs mitomycin C control).

**Table 3.6: Cytotoxic effect of gemcitabine and mitomycin C on UROtsa cells in their non-proliferative and proliferative states, measured immediately, 24, 48 and 72 hours after treatment at 42°C. Data is reported as pLC50 and standard error (n=6), analysed with a comparison of the non-linear curve fits.**

42°C Incubation												
Non-Proliferative UROtsa Cells							Proliferative UROtsa Cells					
Gemcitabine Treatment			Mitomycin C Treatment				Gemcitabine Treatment			Mitomycin C Treatment		
pLC50	Std. Error	p value	pLC50	Std. Error	p value		pLC50	Std. Error	p value	pLC50	Std. Error	p value
24hrs	1.1	2.1	-3.6	0.1			-1.0	0.6		-3.2	0.1	
48hrs	-3.1	0.4	p<0.001	-4.2	0.1	p<0.001	-2.2	0.3	p=0.001	-3.4	0.1	p=0.001
72hrs	-2.4	0.4		-4.5	0.03		-2.3	0.1		-3.5	0.1	

**Table 3.7: Cytotoxic effect of gemcitabine and mitomycin C on RT4 and T24 cells, measured immediately, 24, 48 and 72 hours after treatment at 42°C. Data is reported as pLC50 and standard error (n=6), analysed with a comparison of the non-linear curve fits. Ambiguous regression data was excluded from analysis.**

42°C Incubation												
RT4 Cells							T24 Cells					
Gemcitabine Treatment			Mitomycin C Treatment				Gemcitabine Treatment			Mitomycin C Treatment		
pLC50	Std. Error	p value	pLC50	Std. Error	p value		pLC50	Std. Error	p value	pLC50	Std. Error	p value
24hrs	Excluded		-1.6	0.2			-2.9	0.5		-2.0	0.3	
48hrs	-3.5	0.6	p<0.001	-3.0	0.2	p<0.001	-5.2	0.2	p<0.001	-2.6	0.2	p<0.001
72hrs	-6.1	0.1		-3.6	0.1		-6.2	0.1		-3.0	0.1	



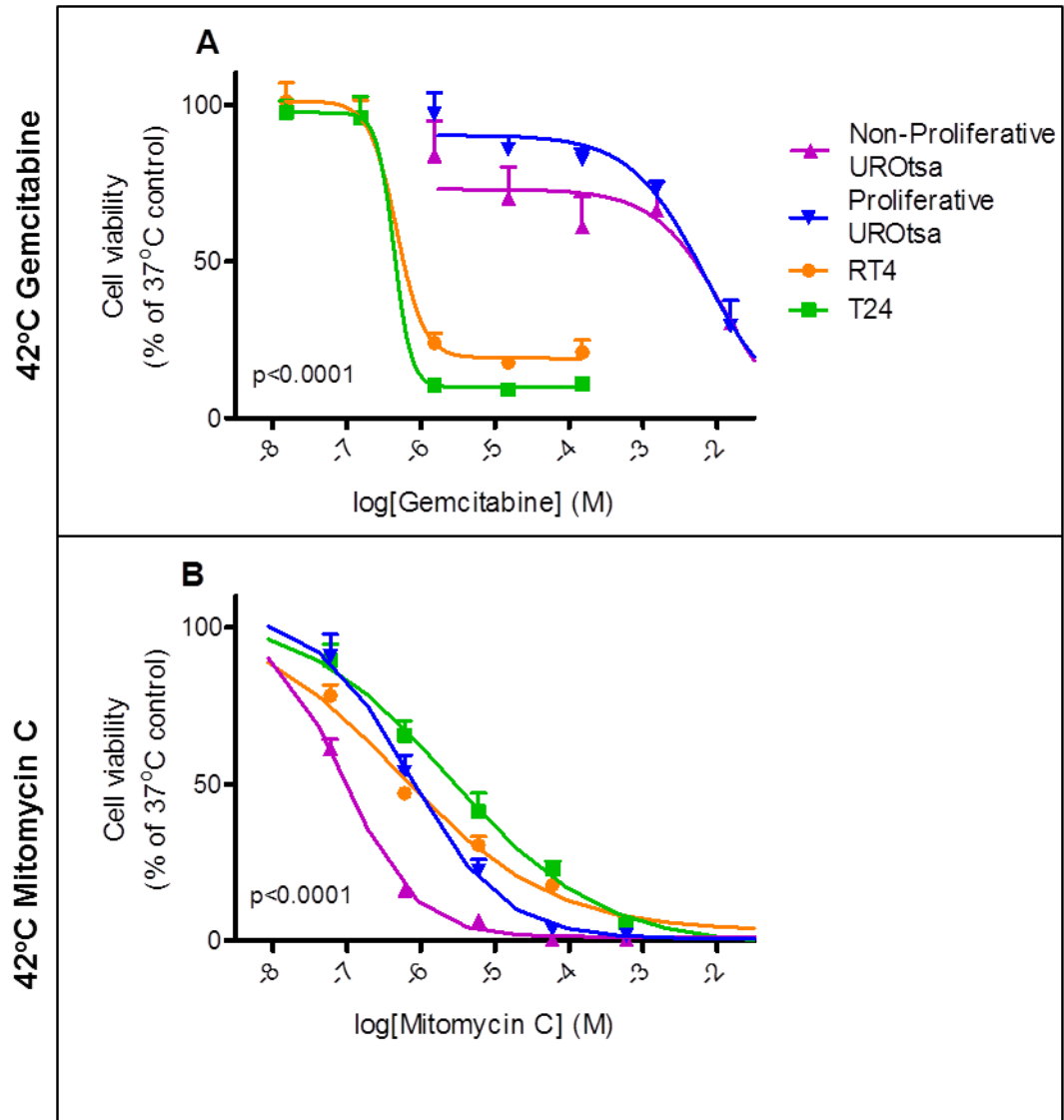


Figure 3.10: Effect of gemcitabine (A) and mitomycin C (B) on urothelial cell viability was measured 72hours after pre-treatment at 42°C. Data are shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ), analysed with a multiple comparisons F-test ( $p < 0.0001$ )

**Table 3.8: LC50 of urothelial cell lines 72 hours post gemcitabine and mitomycin C treatment, incubated at 42°C. LC50s were calculated as mean values of n≥6, compared using an F-test.**

LC50 at 72hours post treatment	Non-Proliferative UROtsa	Proliferative UROtsa	RT4	T24	Comparison of drug cytotoxicity between cell lines
<i>Gemcitabine 42°C (95% CI)</i>	1.2 (0.3-4.9) mM	4.6 (2.5-8.5) mM	0.7 (0.5-1.1) µM	0.6 (0.4-0.9) µM	p<0.0001
<i>Comparison of combined gemcitabine/hyperthermia to gemcitabine alone</i>	P=0.0119	P=0.1239	0.3992	0.1544	
<i>Mitomycin C 42°C (95% CI)</i>	0.1 (0.1-0.1) µM	0.9 (0.6-1.3) µM	0.8 (0.6-1.0) µM	3.0 (1.9-4.8) µM	p<0.0001
<i>Comparison of combined mitomycin C/hyperthermia to mitomycin C alone</i>	p<0.001	p=0.9717	p<0.0001	p=0.0738	
<i>Comparison of cytotoxicity of gemcitabine to mitomycin C on cell lines treated at 42°C</i>	p<0.001	p<0.001	p=0.94	p=0.44	

## PART B – MECHANISMS OF GEMCITABINE TOXICITY

### REACTIVE OXYGEN SPECIES PRODUCTION

The basal levels of reactive oxygen species was measured in the urothelial cell lines 12 hours post seeding (**Figure 3.11**). ROS levels in the malignant urothelial cell lines RT4 and T24 was 1.7- and 2.4-fold greater than the UROtsa cell line in its proliferative state ( $p>0.05$  and  $p>0.0001$  respectively). UROtsa cells in the non-proliferative form produced significantly less ROS under basal conditions than the T24 cells ( $p<0.05$ ), but had significantly greater basal ROS production with comparison to UROtsa cells in the proliferative state ( $p<0.05$ ).

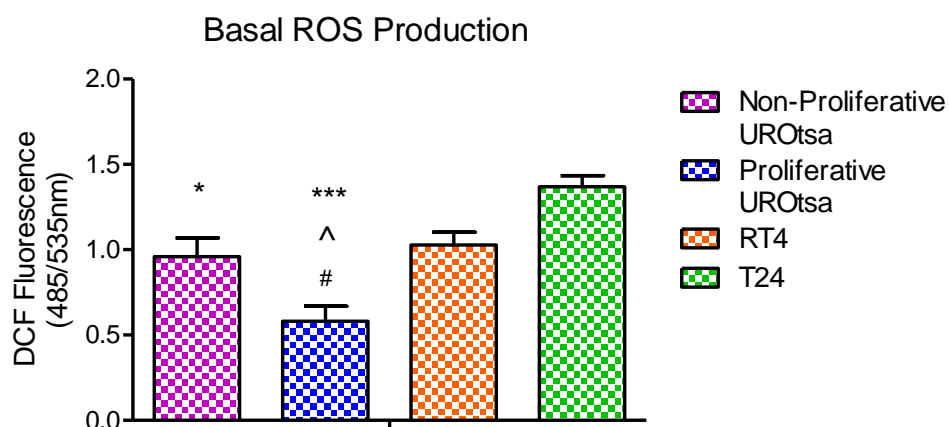


Figure 3.11: Basal reactive oxygen species (ROS) production measured 12 hours post seeding in non-malignant and malignant urothelial cells. Data is shown as DCF fluorescence normalised by cell number (mean  $\pm$  SEM,  $n=6$ ) and analysed using a one-way ANOVA with Tukey-Kramer multiple comparisons test (\* $p<0.05$ , \*\*\* $p<0.001$  vs T24 cells, ^ $p<0.05$  vs RT4 cells, # $p<0.05$  vs non-proliferative UROtsa cells).

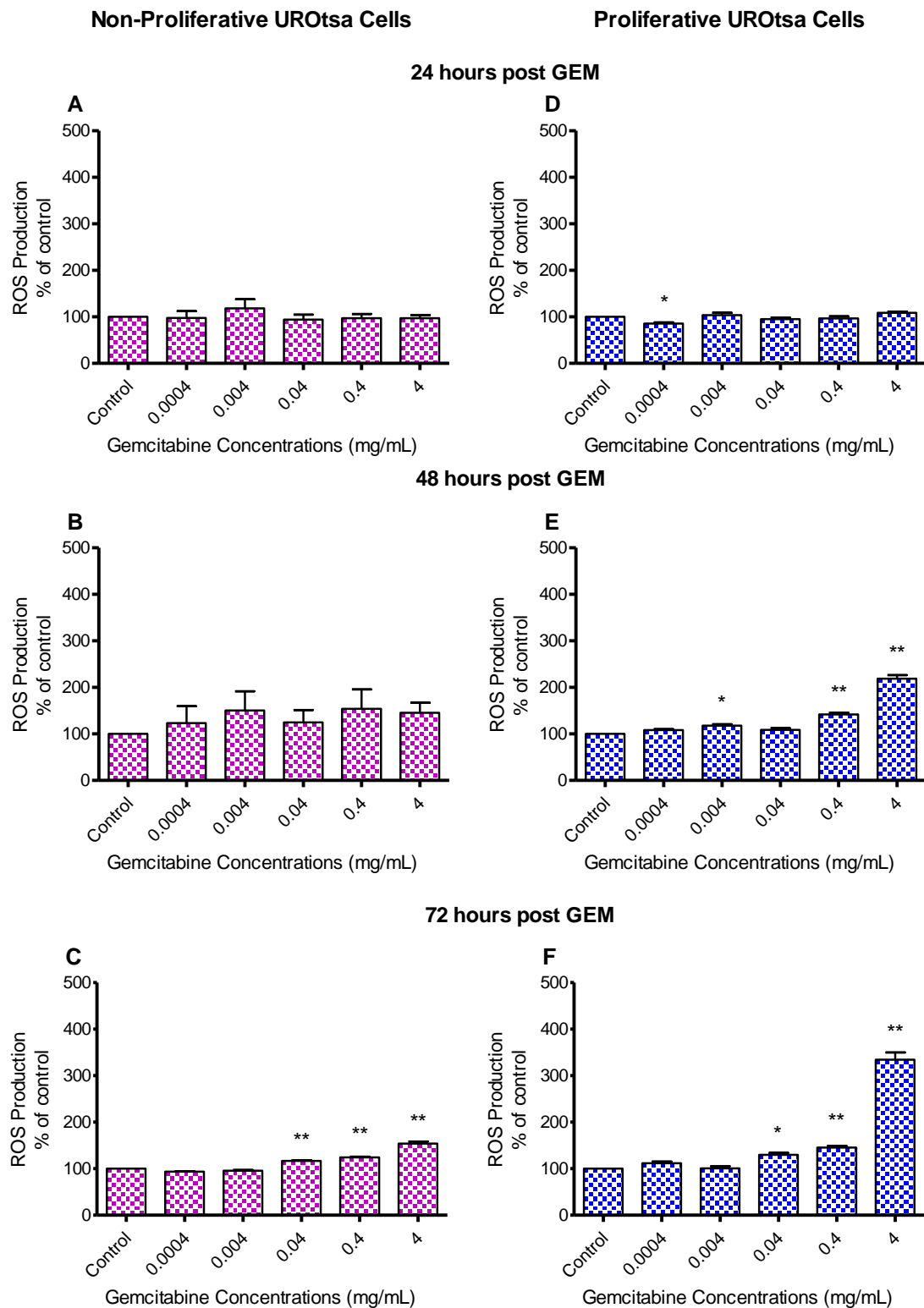
One of the antiproliferative mechanisms of gemcitabine is through the induction of oxidative stress in tumours. ROS formation was also measured after treatment with gemcitabine, 24, 48 and 72 hours post-treatment in all urothelial cell lines. ROS production after gemcitabine treatment tended to increase as cell viability decreased, similarly in a time-dependent manner.

Gemcitabine had no effect on ROS production in non-proliferative UROtsa cells measured 24 and 48hrs post-treatment (**Figure 3.12A&B**). However, treatment (0.04-4mg/mL) caused a 17-54% increase in ROS production in non-proliferative UROtsa cells 72 hours after treatment (**Figure 3.12C**).

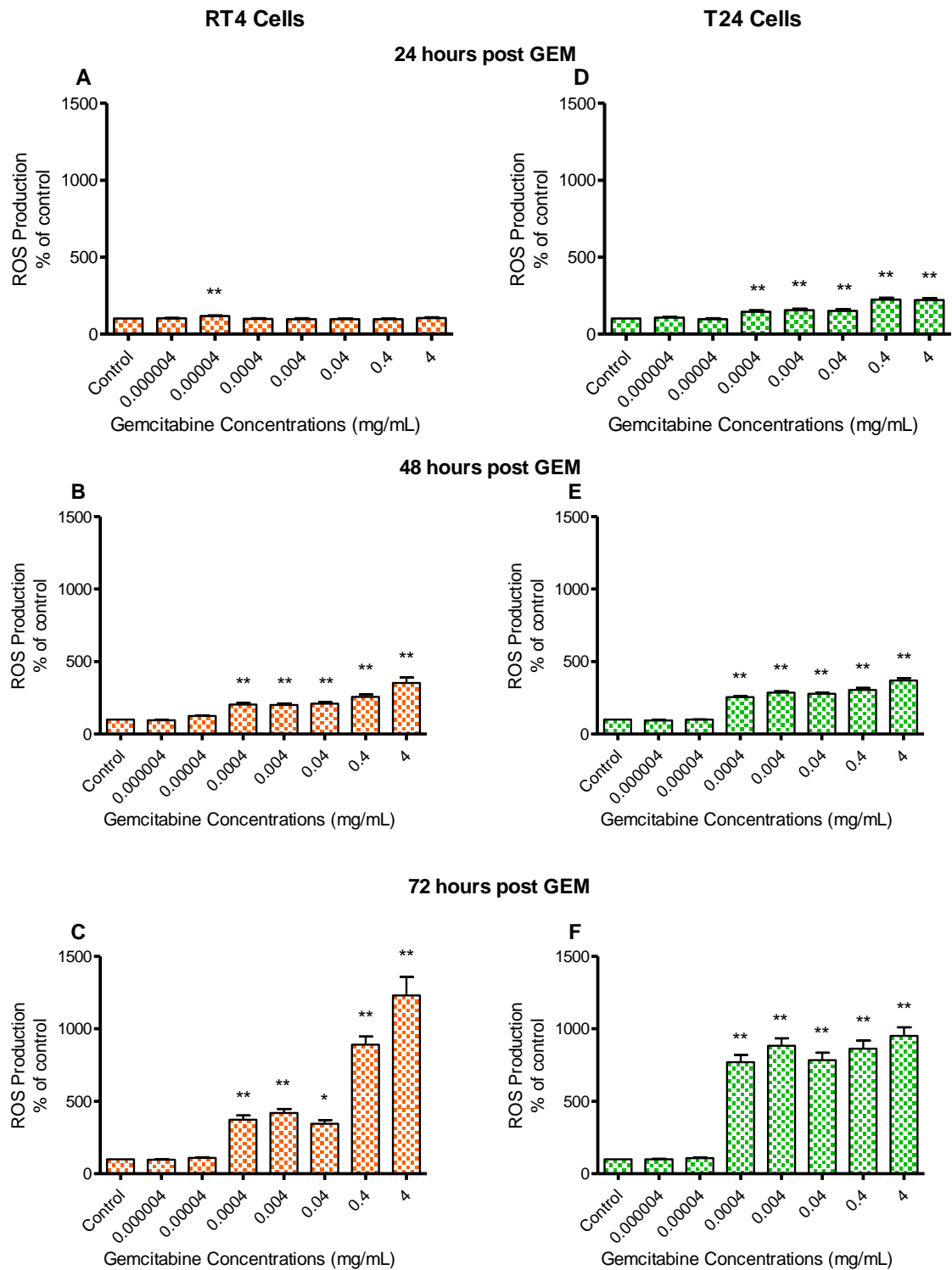
Similarly, gemcitabine did not increase ROS production in proliferative UROtsa cells measured 24 hours post treatment (**Figure 3.12D**). ROS production was significantly enhanced in the proliferative UROtsa cells by 42% and 118% 48 hours after treatment with 0.4 and 4mg/mL (**Figure 3.12E**), and 30%, 45% and 335% 72hrs post treatment with 0.04-4mg/mL (**Figure 3.12F**).

Increased ROS production was noted in RT4 and T24 cells compared to UROtsa cells. ROS production increased in RT4 and T24 cells in a time and concentration dependent manner following gemcitabine treatment. In RT4 cells, ROS production was significantly enhanced by 2-3.5 fold and 3.7-12.3 fold measured 48- and 72hrs respectively after gemcitabine treatment  $\geq 0.0004$ mg/mL (**Figure 3.13 B&C**).

In T24 cells, gemcitabine ( $\geq 0.0004$ mg/mL) induced significantly increased DCF fluorescence ( $p < 0.01$ ) at all time points tested, with an increase up to 9.5-fold at 72hrs post treatment (**Figure 3.13D-F**).



**Figure 3.12: Reactive oxygen species production in UROtsa cells in their non-proliferative (A-C) and proliferative (D-F) states 24 (A&D), 48 (B&E) and 72 (C&F) hours post 1 hour gemcitabine treatment at 37°C. Data is shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ) and analysed using a one-way ANOVA with Dunnett post-test (\* $p < 0.05$ , \*\* $p < 0.01$  vs gemcitabine control).**



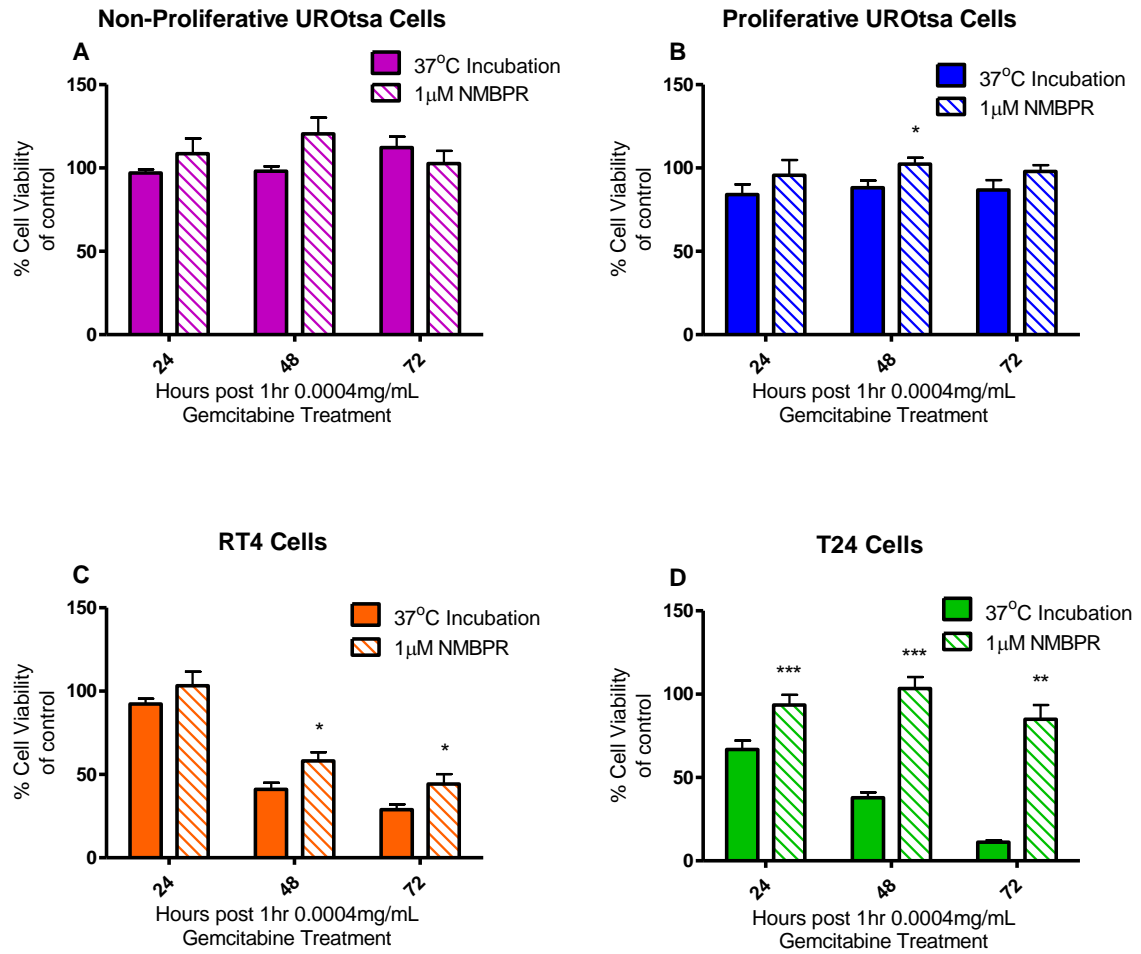
**Figure 3.13: Reactive oxygen species production in RT4 (A-C) and T24 (D-F) cells 24 (A&D), 48 (B&E) and 72 (C&F) hours post 1 hour gemcitabine treatment at 37°C. Data is shown as a percentage of control (mean  $\pm$  SEM,  $n \geq 6$ ) and analysed using a one-way ANOVA with Dunnett post-test (\* $p > 0.05$ , \*\* $p > 0.01$  vs gemcitabine control).**

## CELL VIABILITY IN THE PRESENCE OF NBMPR

The effect of blocking the human equilibrative nucleoside transporter (NBMPR (1 $\mu$ M)) on gemcitabine (0.0004mg/mL) induced changes in cell viability was assessed in each of the urothelial cell lines.

The viability of UROtsa cells in their non-proliferative states were unchanged by concurrent gemcitabine (0.0004mg/mL) and NBMPR (1 $\mu$ M) treatment at all time points tested (**Figure 3.14A**). The cell viability of the proliferative UROtsa cells treated with 0.0004mg/mL gemcitabine were significantly ( $p<0.05$ ) increased in the presence of NMBPR assessed 48hrs, however this was not observed after 72hrs (**Figure 3.14B**).

In comparison, the viability of the cancer cell lines RT4 and T24 treated with 0.0004mg/mL gemcitabine were significantly ( $p<0.05$ ) increased in the presence of NMBPR assessed 48 and 72 hours after treatment (**Figure 3.14 C&D**). The T24 cell line was also protected from the cytotoxicity of gemcitabine at 24 hours. Blocking the human equilibrative nucleoside transporter using NBMPR offered greater protection in T24 cells than in RT4, with cell viability close to control levels in gemcitabine-NBMPR co-treated T24 cells (**Figure 3.14D**).



**Figure 3.14: Effect of 1 hour 0.0004mg/mL gemcitabine treatment with and without the presence of the hENT1-blocker NMBPR on UROtsa cells in their non-proliferative (A) and proliferative (B) states, RT4 (C) and T24 (D) cells, assessed 24, 48 and 72 hours post treatment. Data is shown as a percentage of untreated control (mean  $\pm$  SEM,  $n \geq 5$ ) and analysed using an unpaired students t-test (\* $p > 0.05$ , \*\* $p > 0.01$ , \*\*\* $p > 0.001$  vs gemcitabine treated cells without the presence of NMBPR).**



## PART C – MECHANISMS OF ADVERSE EFFECTS OF GEMCITABINE TREATMENT

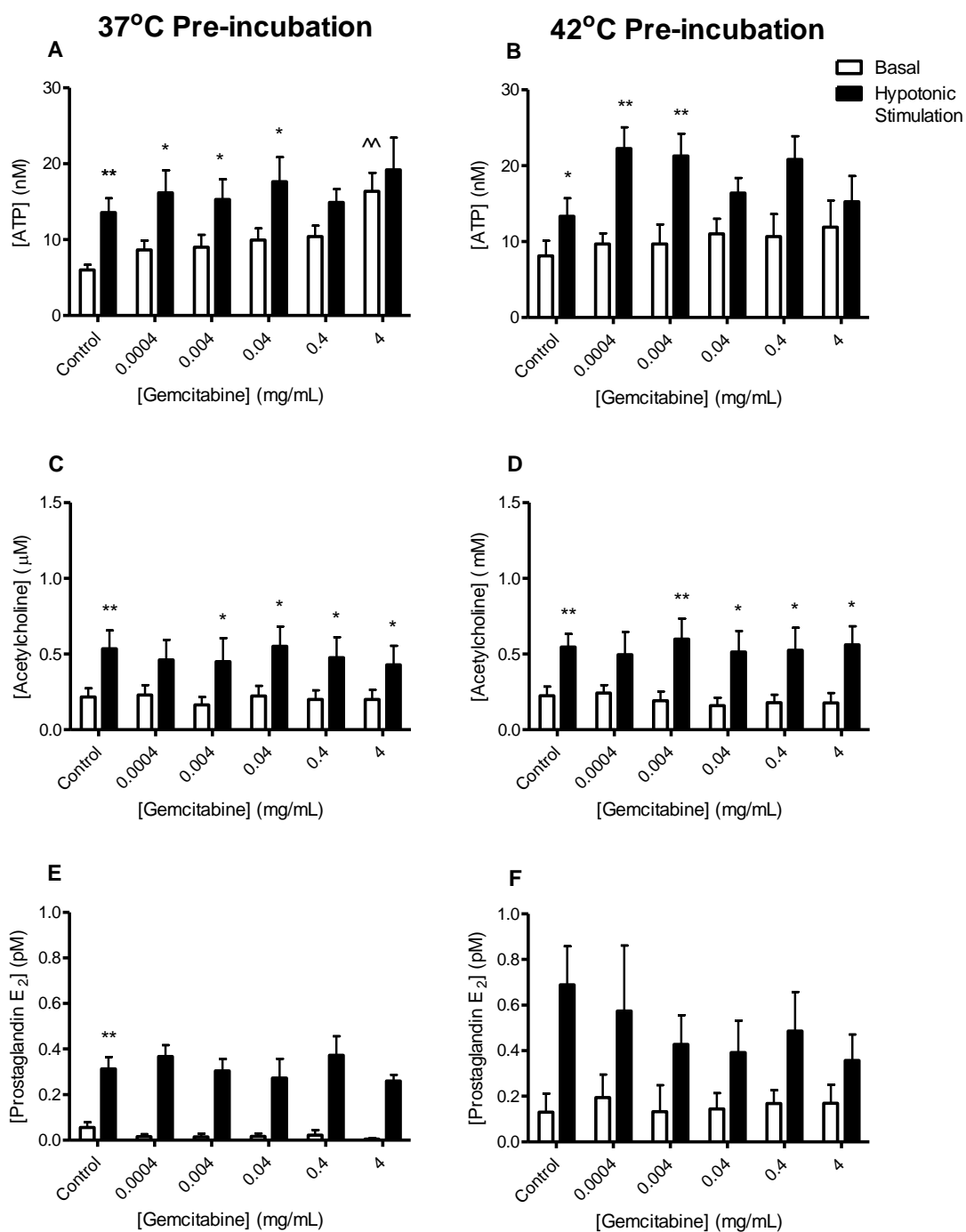
### IMMEDIATE EFFECT OF GEMCITABINE ALONE AND IN COMBINATION WITH HYPERTHERMIA ON UROTHELIAL MEDIATOR RELEASE

Release of mediators from the urothelium of the bladder has been found to play a significant role in coordinating responses of the micturition reflex. Given this role in bladder function, the effect of gemcitabine alone and in combination with hyperthermia immediately post treatment on the basal and stimulated release of mediators from UROtsa cells was investigated.

UROtsa cells release ATP under basal conditions, with a significant increase in release when stretched. Basal release of ATP increased in a concentration dependent manner in gemcitabine treated cells (**Figure 3.15A**), with a significant increase in cells treated with 4mg/mL ( $p<0.01$ ). The increased ATP release with stretch was abolished in UROtsa cells treated with gemcitabine at concentrations  $\geq 0.4$ mg/mL. Pre-incubation of UROtsa cells at 42°C did not change the basal or stretch release of ATP (**Figure 3.15B**), however gemcitabine in combination increased temperature abolished the stretch response of ATP at concentrations  $\geq 0.04$ mg/mL.

Stretch induced release of acetylcholine from control incubated UROtsas was enhanced 2.5 fold from basal release ( $p<0.01$ ). Incubation with gemcitabine had no effect on basal or stimulated release of acetylcholine at 37°C (**Figure 3.15C**). Pre-incubation of UROtsa cells at 42°C had no effect on acetylcholine release, nor did gemcitabine treatment at the increased temperature (**Figure 3.15D**).

Hypotonic stimulation caused a 5-fold increase from basal in prostaglandin E<sub>2</sub> (**Figure 3.15E**). While incubation with gemcitabine did not change the stretch release of prostaglandin E<sub>2</sub>, basal release was decreased to levels below the limits of detection in most of the gemcitabine treatment groups. Therefore, it was not possible to statistically analyse the stretch response in comparison to basal in the gemcitabine groups due to the low basal sample number within the measureable range. Unlike incubation at 37°C, gemcitabine treated UROtsa cells did release prostaglandin E<sub>2</sub> under basal conditions (**Figure 3.15F**), and while an increase in prostaglandin E<sub>2</sub> release in response to stretch was evident, this was not significant at this temperature. Prostaglandin E<sub>2</sub> release was not significantly affected by gemcitabine treatment at 42 °C.



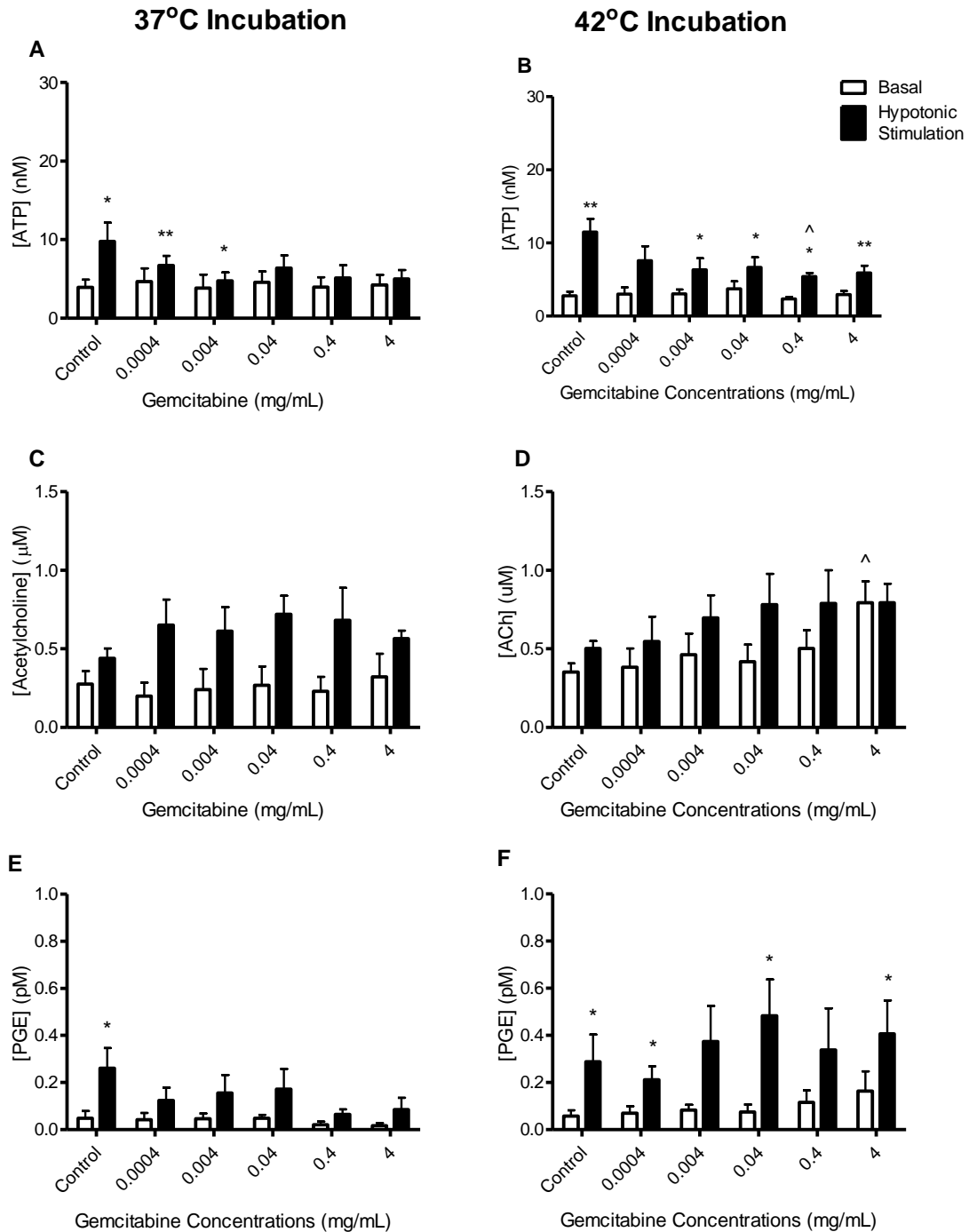
**Figure 3.15: Effect of 1-hour pre-incubation of UROtsa cells with gemcitabine at 37°C (A, C & E) or 42°C (B, D & F) on basal and hypo-osmotically stimulated ATP (A & B), acetylcholine (C & D) and prostaglandin E<sub>2</sub> (E & F) mediator release from UROtsa cells immediately post-treatment. Data shown as the mean ± SEM (n ≥ 4), analysed by a paired two-tailed t-test (\*p > 0.05, \*\*p > 0.01 vs basal release, ) and a one-way ANOVA with Dunnett's post-test (^p > 0.01 vs control).**

## EFFECT OF GEMCITABINE ALONE AND IN COMBINATION WITH HYPERTHERMIA ON UROTHELIAL MEDIATOR RELEASE 24-HOURS POST TREATMENT

Basal release of ATP from UROtsa cells returned to control levels 24 hours after 37°C gemcitabine treatment (**Figure 3.16A**). The stretch response however remained abolished in cells treated  $\geq 0.04$ mg/mL. In contrast, the stretch response was maintained in UROtsa cells treated with gemcitabine at 42°C (**Figure 3.16B**). Stimulated release of ATP decreased in a concentration-dependent manner, with a significant ( $p < 0.05$ ) decrease in cells treated with 0.4mg/mL gemcitabine.

There was no significant difference between the basal and stretch release of acetylcholine from UROtsa cells 24 hours after gemcitabine treatment at either 37°C or 42°C (**Figure 3.16 C&D**). A concentration dependent increase in basal acetylcholine release was observed in UROtsa cells treated at 42°C, significantly ( $p < 0.05$ ) at 4mg/mL gemcitabine (**Figure 3.16D**).

Hypotonic stimulation significantly enhanced ( $p < 0.05$ ) the release of prostaglandin E<sub>2</sub> in control UROtsa cells 24 hours after incubation at 37°C and 42°C (**Figure 3.16 E&F**). Basal prostaglandin E<sub>2</sub> release was measureable in gemcitabine treated samples at 24 hours, unlike those analysed immediately after treatment. The significant stretch response seen immediately after incubation with gemcitabine was abolished after 24 hours at 37°C, but still present in samples from 42°C gemcitabine pre-incubation (**Figure 3.16 E&F**).



**Figure 3.16: Effect of 1-hour pre-incubation of UROtsa cells with gemcitabine at 37°C (A, C & E) or 42°C (B, D & F) on basal and hypo-osmotically stimulated ATP (A & B), acetylcholine (C & D) and prostaglandin E<sub>2</sub> (E & F) mediator release from UROtsa cells 24-hours post-treatment. Data shown as the mean  $\pm$  SEM (n $\geq$ 4), analysed by a paired two-tailed t-test (\*p < 0.05, \*\*p < 0.01 vs basal release) or a one-way ANOVA with Dunnett's post-test (^p < 0.05 vs control).**

## EFFECT OF GEMCITABINE PRE-TREATMENT ON INFLAMMATORY CYTOKINE RELEASE

The release of inflammatory cytokines (Interleukin-1 $\beta$ , -6, -8, -10, 12p70 and tumour necrosis factor) from urothelial UROtsa cells was measured in cell free media 24hrs after 1hr gemcitabine treatment at 37°C or 42°C. Interleukin-6 and interleukin-8 were secreted by control urothelial cells (**Figure 3.17 A&B**), and this was not significantly affected by increased temperature.

Secretion of IL-6 by UROtsa cells was significantly enhanced by 4mg/mL gemcitabine treatment, with an 11- and 10- fold increase from the 37°C and 42°C control incubations to 1816  $\pm$  108 pg/mL and 2628  $\pm$  334 pg/mL respectively (**Figure 3.17A**). The effect of increased temperature (42°C) in combination with gemcitabine treatment significantly enhanced ( $p < 0.05$ ) IL-6 release with comparison to gemcitabine treatment at 37°C.

A significant increase in IL-8 release was also observed after 4mg/mL gemcitabine treatment at 37°C (5.6 fold increase) and 42°C (6 fold) (**Figure 3.17B**).

Interleukin-1 $\beta$  release was not detected in samples from control UROtsa cells, however it was detected in gemcitabine treated samples following incubation at 37°C or 42°C (**Figure 3.17C**).

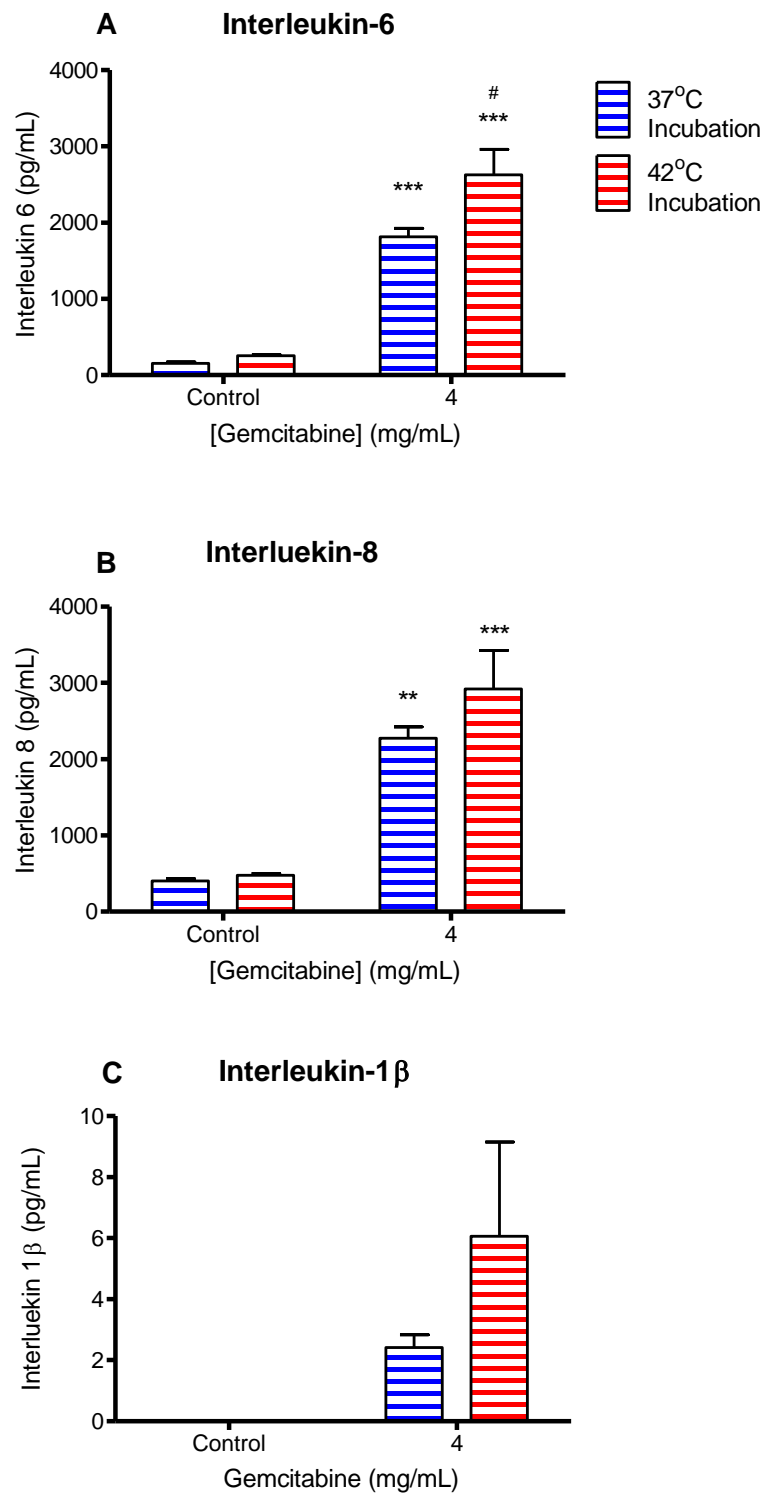


Figure 3.17: Effect of 1-hour gemcitabine incubation at 37°C or 42°C on cytokine release from UROtsa cells 24 hours post-treatment. Data shown as the mean  $\pm$  SEM ( $n \geq 3$ ), analysed by a one-way ANOVA with Bonferroni post-test (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  comparing gemcitabine treated to 37°C control release and #  $p < 0.05$  comparing 37°C and 42°C gemcitabine treated cells).

### **3.5 DISCUSSION**

While gemcitabine has only recently been used in the treatment of bladder cancer, its efficacy and toxicity profile has been shown to be favourable with comparison to other commonly used treatments, including mitomycin C and bacillus Calmette-Guerin therapy (Shelley et al., 2012). To the best of my knowledge, this study is the first to report the selectivity of gemcitabine for cancer vs non-cancer urothelial cell lines, which may explain in part the reported superior toxicity profile.

#### **CYTOTOXICITY OF GEMCITABINE**

The results of this study demonstrated that the cell survival of malignant and non-malignant urothelial cells was reduced by gemcitabine and mitomycin C in a time and concentration dependent manner. Mitomycin C is the most frequently used chemotherapy for non-muscle invasive bladder cancer (NMIBC) (Hendricksen and Witjes, 2007). Numerous studies have investigated the potency of both mitomycin C and gemcitabine on cancer cells, but to the best of my knowledge, this study is the first to report the cell killing effect of these chemotherapeutics on normal urothelial cells.

The cytotoxic actions of both drugs were significantly different between the malignant and non-malignant urothelial cell lines. Gemcitabine was found to be selectively cytotoxic (>10,000 fold) to the cancer cell lines RT4 and T24 with comparison to the UROtsa urothelial cell line. In direct comparison, the cytotoxic actions of mitomycin C were approximately 10-fold more potent on normal urothelial cells compared to bladder cancer cells.

The concentration of mitomycin C is reduced 35-fold across the urothelium (Wientjes et al., 1993). As the clinical dosage for this drug is 2mg/mL, the results of this study indicate that the concentration of mitomycin C penetrating the urothelium of patients would be almost completely cytotoxic to the normal urothelial cells in the bladder (<10% cell viability of non-proliferative and proliferative UROtsa cells). While the penetration ability of gemcitabine across the urothelium is unknown, a concentration similarly reduced 35-fold from the 40mg/mL clinical dose would result

in a reduction in urothelial cell viability by approximately 35-55% (based on interpolation from UROtsa cell viability in **Figure 3.7**). As the primary role of the urothelium is to form a protective barrier for the underlying bladder nerves and muscle from the contents of urine, it could be assumed that this function may be impaired after intravesical treatment with cytotoxic drugs. It has been shown that urothelial damage to the apical umbrella cells and GAG layer induced by intravesical protamine sulphate in rat bladders results in bladder hyperactivity and increased pain sensitivity to noxious stimuli, although the mechanisms behind these changes are still unclear (Okada et al., 2014). These symptoms are similar to those reported by patients undergoing intravesical chemotherapy for NMIBC, and it could be suggested that urothelial damage by cytotoxic drugs and consequent increased permeability of the urothelium is in part responsible for these side effects. Furthermore, as the incidence of reported side effects including dysuria and bladder pain is significantly greater in patients treated with mitomycin C compared to gemcitabine (72% to 39%) (Addeo et al., 2010), the results of the present study may explain this effect.

Previous studies have investigated the cytotoxicity of gemcitabine between both human and rat bladder cancer cell lines and fibroblasts cultured alone or with a TCC as a spheroid co-culture (Kilani et al., 2002). The human bladder tumour cell lines MGH-U3 and RT-112 (derived from human non-invasive TCC bladder tumour and epithelial bladder carcinoma respectively) and rat TCC line AY-27 were selectively killed by gemcitabine treatment with relative sparing of the fibroblast cells studied in *in vitro* spheroid models (Lin et al., 1985, Kilani et al., 2002). This correlates with the results of the present study, where gemcitabine was selectively cytotoxic to malignant bladder cancer cell lines RT4 and T24 with comparison to normal urothelial cells.

The cytotoxic actions of gemcitabine are dependent on several mechanisms. It is considered a prodrug, requiring transport into the cell primarily through nucleoside transporters before being converted to its active forms through the enzymes deoxycytidine kinase (dCK) and thymidylate kinase 2 (TK2). These phosphorylated forms of gemcitabine act by being incorporated into DNA strands, causing masked chain termination and inhibiting ribonucleotide reductase (RNR), dCMP deaminase and DNA polymerase (Gandhi et al., 1996, Candelaria et al., 2010, Cerqueira et al., 2007, Gandhi and Plunkett, 1990). Gemcitabine moves into cells through nucleoside



transporters, acting as a substrate for five of the human equilibrative and concentrative nucleoside transporters, with hENT1 having the greatest affinity for gemcitabine (Mabel W. L. Ritzel, 2001).

The functional effect of concurrent incubation of the nucleoside transporter inhibitor S-(4-nitrobenzyl)-6-thioinosine (NBMPR) with gemcitabine on changes in urothelial cell viability was tested in this study. Concurrent gemcitabine and 1  $\mu$ M NBMPR treatment significantly improved cell survival in the urothelial cancer cell lines, RT4 and T24. Previous work has shown that RT4 cells have a higher number of total NBMPR binding sites, the fastest initial uptake of [ $^3$ H] gemcitabine and largest intracellular accumulation of gemcitabine after 2 hour exposures with comparison to T24 cells (Damaraju et al., 2010). The results presented here confirm the findings of the previous study which concluded that there is no link between basal levels of cell surface NMBPR-sensitive nucleoside transporters and gemcitabine sensitivity, as it found in the present study that T24 cells are more sensitive to gemcitabine than RT4 cells (LC50 0.4[0.3-0.6]  $\mu$ M and 1.0[0.5-2.0]  $\mu$ M respectively) (Rauchwerger et al., 2000). In addition, co-incubation with NBMPR offered greater protection from gemcitabine induced cytotoxicity in T24 cells compared to RT4 cells.

However, there was no protective effect of NMBPR observed in the UROtsa cells measured 72 hours post treatment. Although there is no correlation between basal levels of hENT transporters and gemcitabine sensitivity, further studies have reported that the presence of these transporters is required for gemcitabine toxicity (Mackey et al., 1998). These results suggest that the expression pattern of hENT1s may be significantly reduced in normal urothelial cells with comparison to bladder cancer cell lines RT4 and T24 and therefore play a role in the observed selectivity.

Gemcitabine cytotoxicity is also reliant on the intracellular conversion by dCK and TK2 to its monophosphate form. This is considered to be the rate limiting step to the accumulation of the active forms of gemcitabine di- and tri-phosphate (Plunkett et al., 1995). Quantification of dCK activity found that T24 cells had a greater than twofold higher dCK activity with comparison to RT4 cells (Damaraju et al., 2010). While RT4 cells display a fourfold increase in the number of total hENT1 binding sites, it appears that T24 cells possess a more efficient phosphorylation of

cytidine analogues, which may account for the enhanced cytotoxicity of gemcitabine on these cells seen within this study. TK2 is a mitochondrial enzyme that phosphorylates pyrimidine deoxynucleosides and their analogues to the monophosphate form. However, the substrate specificity of gemcitabine for TK2 is only 5-10% of that of dCK, suggesting gemcitabine toxicity is predominantly reliant of the dCK phosphorylation of gemcitabine (Wang et al., 1999). The dCK/TK2 activity of UROtsa or normal urothelial tissue has not been confirmed within the literature, and may be a further mechanism for the resistance to gemcitabine observed in UROtsa cells in this study.

### **CYTOTOXICITY OF GEMCITABINE AND HYPERTHERMIA**

Hyperthermia (42°C) did not enhance the cell killing effect of gemcitabine in RT4, T24 or proliferative UROtsa, however, a significant increase in the cytotoxicity was observed in the non-proliferative UROtsa cells with comparison to incubation at 37°C (LC50 1.2[0.3-4.9]mM to 11.5[4.5-28.9]mM respectively). This result is in contrast to previously reported gemcitabine and hyperthermia synergism on T24 and RT4 cells, however cell death was measured in that study only 24 hours after 1 hour gemcitabine treatment at hyperthermic conditions, which may account for the variance in this result (van der Heijden et al., 2005). As the effects of hyperthermia *in vivo* are multifactorial, including injury to tumour vasculature and increased immune response, combined hyperthermia and gemcitabine treatment may have therapeutic benefits in patients that cannot be observed in a simple urothelial cell culture model (Rampersaud et al., 2010).

This study found that hyperthermia enhanced the cytotoxicity of mitomycin C on non-proliferative UROtsas and the cancer cells RT4 and T24. This result correlates with previous studies finding enhanced cytotoxicity of mitomycin C and hyperthermia on cancer cells (van der Heijden et al., 2005).

UROtsa cells grown in serum supplemented media have been found to express the basal patterns of metallothionein and heat shock proteins 27, 60 and 70, in agreement with that known to occur in *in situ* urothelium (Somji et al., 2001, Lebreton et al., 2003, Rossi et al., 2001).

Rossi and colleagues (2001) found that UROtsa cells grown in serum-free media expressed significantly reduced levels of Hsp 70 with comparison to UROtsa cells grown in serum-supplemented media. Hsp70 has been reported to block apoptosis, and its down-regulation, inhibition or under-expression can facilitate the induction of apoptosis (Nylandsted et al., 2000, Burkart et al., 2000). Exposure of cells to temperatures of 42°C activates Hsp70 and its apoptotic-protective effect (Abravaya et al., 1992). This may explain enhanced cell death in non-proliferative UROtsa cells (grown in serum-free media) with increased temperature and gemcitabine/mitomycin C, while no change was observed in the proliferative UROtsa cells (grown in serum-supplemented media) treated at the same conditions.

## **GEMCITABINE AND THE INDUCTION OF REACTIVE OXYGEN SPECIES**

With comparison to normal cells, cancer cells experience greater oxidative stress associated with oncogenic stimulation, altered metabolic activity and increased reactive oxygen species (Pelicano et al., 2004). In line with this observation, this study found that the cancer cell lines T24 and RT4 have a significantly greater basal ROS production with comparison to non-cancer UROtsas cells.

However, cancer cells that are exposed to persistent intrinsic ROS stress adapt to these mechanisms by activating transcription factors including nuclear factor kappa-light-chain-enhancer of activated B cells and hypoxia-inducible factor-1 $\alpha$  (Pervaiz and Clement, 2004, Sullivan and Graham, 2008, Tiligada, 2006). This in turn leads to enhanced activation of the antioxidant defence system including superoxide dismutase and promotes expression of cell survival proteins, a mechanism that has been observed in bladder cancer cell lines (Gupta et al., 2012, Hour et al., 2004).

ROS production induced by gemcitabine has been associated with its cytotoxicity, through increasing mitochondrial membrane permeability and promoting apoptosis (Donadelli et al., 2007). The inherent basal production of reactive oxygen species of pancreatic adenocarcinoma cells and sensitivity to gemcitabine has been compared, implicating cell lines with a lower basal level of ROS to be more resistant to gemcitabine (Donadelli et al., 2007). However, it has

recently been reported that pancreatic cancer cell lines resistant to gemcitabine also have enhanced antioxidant mechanisms, including glutathione (GSH) production (Ju et al., 2015). However, Ju et al. (2015) found that reducing GSH production by the addition of  $\beta$ -phenylethyl isothiocyanate inhibited the ROS antioxidant mechanism. This may provide a future treatment avenue for bladder cancer cells resistant to gemcitabine induced ROS-mediated cell death.

The bladder cancer cell lines RT4 and T24 treated with gemcitabine displayed greater than 10-fold increase in ROS production from controls, with comparison to a less than 3-fold ROS production in the normal bladder epithelial UROtsa cells after treatment with gemcitabine. The resistance of the normal urothelial cells to the cytotoxic effect of gemcitabine may in part be explained by the lower basal levels of ROS, or an antioxidant mechanism capable of mitigating the ROS-induced effects in comparison to the cancer cell lines.

## **MECHANISMS OF ADVERSE EFFECTS AFTER GEMCITABINE TREATMENT**

While intravesical therapy for bladder cancer limits systemic absorption of chemotherapies, the urothelium lies in direct contact with cytotoxic drugs. Insult to the urothelium from these therapies may explain the commonly reported urological side effects, including dysuria, increased frequency and urgency of urination, suprapubic pain and cystitis (Thrasher and Crawford, 1992, Bolenz et al., 2006). Gemcitabine treatment for bladder cancer has reported to have a better toxicity profile and fewer reported side effects with comparison to other chemotherapies, including mitomycin C, epirubicin and doxorubicin (Shelley et al., 2012). A randomized Phase III trial reported the incidence of adverse effects in only 39% of the patient cohort treated with gemcitabine in comparison to 72% of patients treated with mitomycin C (Addeo et al., 2010).

As the urothelium is in direct contact with cytotoxic drugs during intravesical therapy, it is likely that the commonly reported side effects of dysuria, increased frequency and urgency of urination and in some cases subpubic pain are in part due to the changes in release of urothelial mediators. Doxorubicin, a commonly used chemotherapeutic for bladder cancer, has been reported to enhance stretch induced release of prostaglandin  $E_2$  and inflammatory

cytokines interleukin-8 and -1 $\beta$  from urothelial cells, which may explain the symptoms of bladder dysfunction experienced in patients (Kang et al., 2013a). It has also recently been shown that mitomycin C induces inflammatory cytokine and nitric oxide release from urothelial cells which persist after recovery periods (Kang et al., 2015).

This study found that UROtsa cells release ATP under basal conditions, and release was significantly enhanced with stretch, in line with previous studies investigating mediator release from UROtsa cells (Ochodnický et al., 2013, Mansfield and Hughes, 2014b). UROtsa cells treated with gemcitabine at 37°C had significant increase in basal ATP release immediately after treatment. Urothelial ATP acts on the purinergic receptors in the A $\delta$  sensory nerve fibres to initiate the micturition reflex and on high threshold C fibres to induce perception of pain (Burnstock, 2009). Commonly reported side effects after intravesical gemcitabine treatment include subpubic pain and dysuria (Addeo et al., 2010), which may be a result of the increased ATP release from the urothelium. However, this enhanced basal ATP release was transient and was not observed 24 hours post treatment, indicating recovery of urothelial ATP release mechanisms. Similarly, Kang et al. (2015, 2013a) found that mediator release recovered over time in doxorubicin and mitomycin treated urothelial cells.

Similarly, UROtsas incubated at 42°C released significantly enhanced ATP levels with hypotonic stimulation. However, after twenty-four hours of recovery from treatment, combined gemcitabine and hyperthermia treated cells released significantly less ATP with hypotonic stimulation. This may suggest the pain reported by patients may be due to sensitization of bladder C afferent nerves rather than enhanced ATP release, which has been observed painful bladder syndrome and other bladder disorders (Kumar et al., 2005). However, in the intact bladder, urothelial cells are not the only source of ATP, as myofibroblasts found in the sub-urothelial layer have been shown to release ATP (Cheng et al., 2011). The close contact of myofibroblasts to nerves within the bladder may implicate their role in mechanosensation of the bladder (Wiseman et al., 2003), and it is unknown as to the effect that gemcitabine has on these cells.

UROtsa cells have the components necessary for the synthesis of acetylcholine and express muscarinic receptors (Arrighi et al., 2011). It was found in this study that UROtsa cells release acetylcholine under basal conditions, and this release was enhanced with stretch. The role of

urothelial derived acetylcholine is uncertain but it may influence sensory nerve activity or stimulate the underlying detrusor muscle, acting in an autocrine and paracrine manner on the urothelium itself (Daly et al., 2010, Hanna-Mitchell et al., 2007). A study by Kanai et al. (2007) reported that muscarinic receptor stimulation on strips of rat bladder released intracellular  $\text{Ca}^{2+}$  and induced membrane-potential transients which originate at the urothelial-suburothelial interface, spreading to the detrusor muscle. Furthermore, they found that enhanced spontaneous activity was inhibited with atropine, thus suggesting that acetylcholine released from the urothelium plays a role in spontaneous, non-voiding contractions of the bladder (Kanai et al., 2007).

From the present study, it appears that gemcitabine, increased temperature or combined gemcitabine and hyperthermia therapy has no effect on the cholinergic mechanisms of the bladder urothelium measured immediately after treatment. There was no significant difference between acetylcholine released under basal and stretched conditions 24 hours after gemcitabine, although this may be due to changes in cellular components due to serum starvation. Interestingly, basal acetylcholine release increased in a concentration dependent manner 24 hours after incubation of UROtsa cells treated with gemcitabine at  $42^{\circ}\text{C}$ , significantly at  $4\text{mg/mL}$ . Similarly, mitomycin C enhanced basal release of acetylcholine from urothelial cells after a twenty-four hour period of recovery (Kang et al., 2015). Hence, increased release of basal acetylcholine from urothelial cells may influence the underlying detrusor muscle tone and enhance spontaneous contractions of the bladder mediated by the muscarinic receptors, a trait of unstable bladders from both humans and animals (Steers, 2002). This may explain in part the symptoms of bladder overactivity reported by patients undergoing intravesical therapy with these agents.

Prostaglandin  $\text{E}_2$  is released by the urothelium with mechanical stretch and is believed to stimulate release of ATP from urothelial cells, and modulate afferent nerve activity and efferent neurotransmission, potentiated by obstructive and overactive bladder disorders (Pinna et al., 2000, Anderson, 1993, Tanaka et al., 2011). Prostaglandin  $\text{E}_2$  release from control UROtsa cells were measured basally and after hypotonic stimulation, both immediately and twenty-four hours after vehicle treatment. While basal release of prostaglandin immediately after incubation was

below detectable levels in gemcitabine treated (37°C) samples, there was measureable release from UROtsa cells with hypotonic stretch, which was not significantly different from control cells. The decrease in basal prostaglandin E<sub>2</sub> release may be a compensatory mechanism to desensitize afferent nerves in order to reduce the impact increased basal ATP might have on sensory activity. Twenty-four hours after treatment, UROtsa cells treated with gemcitabine released prostaglandin E<sub>2</sub> basally, with no significant stretch response in 37°C incubated cells. An enhanced-stretch response was measured from cells treated at 42°C, comparably to controls. This result is in contrast to the effect of doxorubicin on urothelial cells, which enhanced stretch release of prostaglandin E<sub>2</sub> from urothelial cultures 24 hours post-treatment, and could explain reduced incidence of patient bladder toxicity with intravesical gemcitabine compared to doxorubicin given the role of prostaglandin E<sub>2</sub> in sensitizing bladder afferents (Kang et al., 2013a).

Elevated levels of inflammatory cytokines have been found in the urine of patients with cystitis and overactive bladder syndrome (Tyagi et al., 2010). Altered visceral sensations from the bladder in these conditions have been suggested to be in part due to increased cytokine expression (Gonzalez et al., 2014). Pro-inflammatory cytokines have been implicated in the sensitization of bladder afferents by altering voltage-gated K<sup>+</sup> channel expression, possibly contributing to increased bladder afferent neuron excitability and leading to enhanced sensations of pain (Cheng and Ji, 2008, Hayashi et al., 2009). Furthermore, Heinrich et al. (2011) found that enhanced cytokines modified cell-cell-communication mediated by gap junctions in human bladder smooth muscle cells and suburothelial myofibroblasts, indicating a possible role in the etiology of the overactive bladder.

Specifically, elevated levels of interleukin-6 have been suggested to indicate the severity of inflammation and correlate with the degree of pain in patients with overactive bladder (Lotz et al., 1994, Erickson et al., 2002). While interleukin-8 was originally implicated as a mediator of the inflammatory process, it also plays a role as a growth and essential survival factor for normal human urothelial cells, mediated through the protein kinase B (Akt) pathway (Dobrev et al., 2006, Tseng-Rogenski and Liebert, 2009). The interleukin-1 cytokine family is associated with acute and chronic inflammation, interleukin-1 $\beta$  in particular being associated with a pro-

inflammatory response and implicated in the pathogenesis of haemorrhagic cystitis (Dinarelli, 2011, Macedo et al., 2012, Ribeiro et al., 2002). The 10-fold increase in interleukin-6 and -8 and presence of interleukin-1 $\beta$  from urothelial cells treated with gemcitabine may account for the reported side effects of overactive and painful bladder associated with intravesical gemcitabine treatment for bladder cancer. Furthermore, as the present study has shown that gemcitabine is cytotoxic to urothelial cells, it is likely that the enhanced IL-8 release plays a role in urothelial proliferation to restore the intact barrier. Urothelial cells treated with the chemotherapeutic agent doxorubicin similarly released enhanced cytokines IL-8 and IL-1 $\beta$ , implicating a common role of the inflammatory response in the reported bladder dysfunction by patients undergoing intravesical chemotherapy (Kang et al., 2013a). Mitomycin C also induced IL-8 release from urothelial cells, at a substantially greater fold-change compared to doxorubicin or gemcitabine (Kang et al., 2015). Furthermore, Kang et al. (2015) found that the enhanced IL-8 release persisted after two weeks of recovery, which may account for the increased severity of side effects reported with intravesical treatment using this agent in comparison to gemcitabine (Addeo et al., 2010)



## CONCLUSION

While the side effects from intravesical gemcitabine treatment include dysuria, subpubic pain and overactive bladder, the frequency of these symptoms reported in patients are significantly less than other commonly used cytotoxic agents, including mitomycin C (Addeo et al., 2010). This is the first investigation that shows selectivity of gemcitabine for malignant cancer cells with comparison to non-malignant urothelial cells, with no synergism observed in combined gemcitabine and hyperthermia therapy except on non-proliferative UROtsa cells. This selective cytotoxicity may be in part explained by the decreased presence of hENT1 receptors in normal urothelial cells, and the lower levels of reactive oxygen species production in non-malignant urothelial cells post gemcitabine treatment. In addition, the minor changes and consequent recovery and inhibition of ATP, acetylcholine and prostaglandin E<sub>2</sub> release from urothelial cells after gemcitabine treatment at both 37°C and 42°C may account for the less frequent adverse effects with comparison to other commonly used chemotherapeutics such as mitomycin C and doxorubicin. The most likely explanation for the bladder dysfunction and associated pain after intravesical gemcitabine in bladder cancer patients would be from the induction of inflammatory cytokines interleukin-6, -8 and -1 $\beta$  and the consequent sensitization of bladder nerves.

The results of the present study also form a solid compilation of basic evidence supporting the use of gemcitabine in patients in preference to the current first-line chemotherapy, mitomycin C. Comparatively, gemcitabine is significantly less and more cytotoxic than mitomycin C to normal and malignant urothelial cells respectively. In addition, gemcitabine induces recoverable changes to urothelial mediator release and stimulates a smaller fold-change in cytokine production in comparison to those reported for MMC by Kang et al (2015).





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## **CHAPTER 4:**

# **EFFECTS OF GEMCITABINE ON THE FUNCTION OF ISOLATED PORCINE BLADDER**

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## **4.1 INTRODUCTION**

The previous chapter investigated the effect of gemcitabine and hyperthermia treatment at a cellular level. While cell culture is a useful model to examine the mechanisms of gemcitabine cytotoxicity on urothelial cells, the bladder is a multi-layered organ made up of urothelium, lamina propria and detrusor muscle. Normal functioning of the bladder requires complex communication and interaction between the various cell types, and coordination of these layers. This chapter investigates the effect of luminal gemcitabine and/or hyperthermia on the normal functioning of intact bladder tissue and the isolated urothelium/lamina propria and detrusor layers.

### **PORCINE BLADDER AS A MODEL OF HUMAN TISSUE**

Porcine bladder is frequently used as a model of the human urinary bladder. Multiple studies have reported the resemblance of the anatomy and physiology of the porcine bladder to human, including similarities in neural control, urodynamic and structural characteristics (Crowe and Burnstock, 1989, Sibley, 1984, Parsons et al., 2012).

As described in Chapter 1, the urothelium of human bladders is protected by a glycosaminoglycan layer. Immunofluorescence studies in porcine urothelium have confirmed the presence of a GAG-layer of similar expression and distribution to human (Janssen et al., 2013). The family of uroplakins (including U1a, U1b, U11 and U111) present in the human asymmetrical unit membrane (AUM) similarly form the majority of the protein component in the urothelial plaques in porcine urothelium (Wu et al., 1994). Furthermore, cytokeratin 20, a protein expressed by umbrella and differentiated intermediate cells in the human urothelium, and cytokeratin 7, expressed by all cells of the urothelium, are similarly present in both human and porcine species (Southgate et al., 1999, Turner et al., 2008).

Rhythmic spontaneous activity from bladder strips originating in the trigone and dome sections of porcine tissues has been found to be comparable in both frequency and amplitude to that in humans (Sibley, 1984). Spontaneous activity of the bladder is influenced by the presence of the urothelium, and has been confirmed to be myogenic in origin, dependent on calcium entry

through voltage-dependent  $\text{Ca}^{2+}$  channels and release from the sarcoplasmic reticulum through ryanodine receptors (Buckner et al., 2002, Brading, 1997, Akino et al., 2008). The role of this activity has been suggested to allow individual muscle bundles to adjust their length in response to filling (Brading, 2006). However, isolated porcine urothelium/lamina propria also displays spontaneous contractile activity, regulated by acetylcholine and M3 muscarinic receptors (Moro et al., 2011).

Activation of the detrusor smooth muscle in the micturition reflex is primarily mediated by the release of acetylcholine from parasympathetic nerves acting upon muscarinic receptors. While the expression of muscarinic subtypes is species specific, the density of muscarinic receptors in human detrusor was found to be similar to porcine detrusor (Chess-Williams, 2002). M2 and M3 receptors are present in a 3:1 ratio in both human and porcine bladder, with the M3 receptor primarily responsible for mediating tissue contractility, stimulating release of intracellular  $\text{Ca}^{2+}$  (Fetscher et al., 2002, Yamanishi et al., 2000, Chess-Williams, 2002). Furthermore, the inhibitory effect of the urothelium on the detrusor muscle through the release of UDIF has been shown in both human and porcine tissue in response to both endogenous muscarinic stimulation and efferent nerve mediated responses (Chaiyaprasithi et al., 2003, Hawthorn et al., 2000).

As discussed in Chapter 1, ATP is released from both the human and animal bladder in response to stretch and other stimuli (Ferguson et al., 1997). Recently, ATP has been found to be released spontaneously and this is enhanced with hypotonic stretch by urothelial, myofibroblast cells and detrusor muscle cells isolated from porcine tissues (Cheng et al., 2011). ATP has been implicated as a mediator for the non-adrenergic, non-cholinergic (NANC) contractions of the bladder, where it is released from nerves and acts upon G-protein-coupled P2Y and ionotropic P2X receptors, inducing contraction by opening nonselective cation channels causing depolarisation and  $\text{Ca}^{2+}$  entry through L-type  $\text{Ca}^{2+}$  channels and subsequent intracellular release (Burnstock, 2007). Furthermore, it is well established that the characteristics of ATP release from porcine and human bladders are similar (Kumar et al., 2004).

Noradrenaline is released by the sympathetic nerves of the bladder and binds to the  $\alpha$ - and  $\beta$ -adrenoceptors found in the bladder base and detrusor muscle respectively. Activation of the  $\alpha$ -

adrenoreceptors cause contraction of the bladder base/trigone, preventing urine leakage, and stimulation of the  $\beta$ -adrenoreceptors induce relaxation of the detrusor muscle to accommodate bladder filling. Similar to human, the  $\beta_3$ -subtype is the most populous of the adrenoreceptors found in the porcine bladder (Yamanishi et al., 2002b, Nomiya and Yamaguchi, 2003). Relaxation to the adrenergic agonist isoprenaline is similarly mediated via the  $\beta_3$ -AR in both human and pig species (Igawa et al., 1999, Igawa et al., 1998, Takeda et al., 1999, Yamanishi et al., 2002b, Yamanishi et al., 2002a).

Electrical field stimulation of bladder nerves induces contraction of the detrusor mediated through the post-ganglionic release of acetylcholine and ATP. Investigation of the electrical properties of the detrusor smooth muscle from pig and human bladder found similarities in the contractile responses, which make them a suitable model to investigate human bladder function (Hashitani and Brading, 2003). These included similarities between species in the fast after-hyperpolarization of action potentials and a dependence on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  and voltage-dependent  $\text{K}^+$  channels on the regulation of electrical activity of the detrusor smooth muscle.

## **INTRAVESICAL GEMCITABINE: EFFECT ON STRUCTURE AND FUNCTION OF THE BLADDER**

Numerous studies have investigated the pharmacokinetics and tolerability of intravesical gemcitabine for bladder cancer. Common urological side effects of treatments in humans of doses between 500-2000mg/50mL include increased urinary frequency and urgency of urination, haematuria, dysuria and in some cases, interstitial cystitis and subpubic pain (Dalbagni et al., 2002, Laufer et al., 2003, Witjes et al., 2004, De Berardinis et al., 2004).

A study of repeated instillations (once per week for 6 weeks) of 175 and 350mg (in 50mL 0.9% saline) gemcitabine in pigs reported no systemic absorption, bladder toxicity or systemic toxicity (Witjes et al., 2003). Thickening of the urothelial layer was observed after 6 instillations of 350mg gemcitabine in the porcine bladder. A preclinical study in beagle dogs of three instillations per week for 4 weeks of 1g gemcitabine (in 50mL saline) similarly observed urothelial thickening due to haemorrhage and cystitis in histological sectioning of the treated



bladders (Cozzi et al., 1999). While various dose regimes and concentrations have been investigated within animal studies, intravesical gemcitabine at 40mg/mL has been reported as a tolerable and effective dose in human patients with bladder cancer and is hence used within this study (Addeo et al., 2010, Mattioli et al., 2005).

To the best of my knowledge, no studies have investigated the effect of gemcitabine on the normal function of the bladder, and the aetiology of the reported side effects after intravesical gemcitabine. The previous chapter found urothelial cells treated with gemcitabine transiently released enhanced levels of basal ATP with comparison to control cells, with little change in release of acetylcholine and prostaglandin E<sub>2</sub>. However, the urothelium/lamina propria in the bladder form a complex network comprised of urothelial cells in various states of differentiation and myofibroblasts, the latter of which have also been found to release ATP spontaneously and in response to stretch (Cheng et al., 2011). Changes in mediator release from the urothelium/lamina propria have been implicated in many pathological conditions of the bladder, as discussed in Chapter 3. Additionally, the effect of gemcitabine on detrusor muscle function will be examined within this study.

## **EFFECT OF HYPERTHERMIA ON THE BLADDER**

Hyperthermia treatment has been extensively investigated as an adjunct treatment with chemotherapy for cancer. A review of treatment with mitomycin C and localised hyperthermia treatment in bladder cancer patients has reported significant benefits in recurrence of NMIBC (Lammers et al., 2011). While there is a significant place for hyperthermia in cancer treatment, little investigation has been performed on the thermal pathology of this treatment (Fajardo, 1984). Combined mitomycin C and hyperthermia is associated with increased adverse effects in patients manifesting as urological symptoms (including urgency, haematuria and pain) compared to treatment with mitomycin C alone, however this has not been reported as statistically significant (Lammers et al., 2011, Colombo et al., 1996). Endoscopic and histologic features of combined mitomycin C and hyperthermia have shown selective damage to the neoplastic area with minimal changes in the normal urothelium (Rigatti et al., 1991).

The effect of hyperthermia on the bladder wall was investigated in dogs, and no gross or microscopic alterations with irrigation of water at temperatures between 35-44.5°C were found (Netto et al., 1973). Similarly, a study of 1-hour intravesical treatment with water at temperatures between 41-43°C in rats found no reduction in bladder capacity (Haveman et al., 2003). Incubation temperatures of 44°C induced increased blood urea nitrogen and transient decreases in bladder volume, while 1 hour bladder hyperthermia at 45°C resulted in the death in 4 (out of 7) rats within a week of treatment. No functional studies using porcine tissue have investigated the effect of hyperthermia on the bladder, or a combination with the chemotherapeutic gemcitabine.

Thus, this study examined the effect of luminal gemcitabine and/or hyperthermia treatment on the structure and function of the porcine bladder. To simulate intravesical treatment, a technique recently described by Smith et al. (2014) was employed. This involves mounting full thickness bladder sections in a bath, exposing the adventitial side in Krebs-bicarbonate solution and applying gemcitabine (40mg/mL) to the urothelial side. This technique allows the selective treatment of urothelium of the bladder, mimicking intravesical treatment in human bladder cancer patients. Consequent dissection of the intact porcine tissue into isolated detrusor and urothelium/lamina propria strips was used as a model to study the effect of gemcitabine on bladder function (Moro et al., 2011, Yamanishi et al., 2000, Yamanishi et al., 2002b).

## 4.2 Aims

The aim of the present study was to investigate the effect of gemcitabine and hyperthermia treatment on the structure and function of the porcine bladder. Specific aims were:

1. To examine the effect of gemcitabine and/or hyperthermia on the urothelium/lamina propria structure
2. To determine the effect of gemcitabine and/or hyperthermia on urothelial mediator release
3. To investigate whether gemcitabine and/or hyperthermia effect changes in the contractility and relaxation of the urothelium and detrusor muscle
4. To determine the effect of gemcitabine and/or hyperthermia on the efferent nerve mediated responses on the detrusor muscle

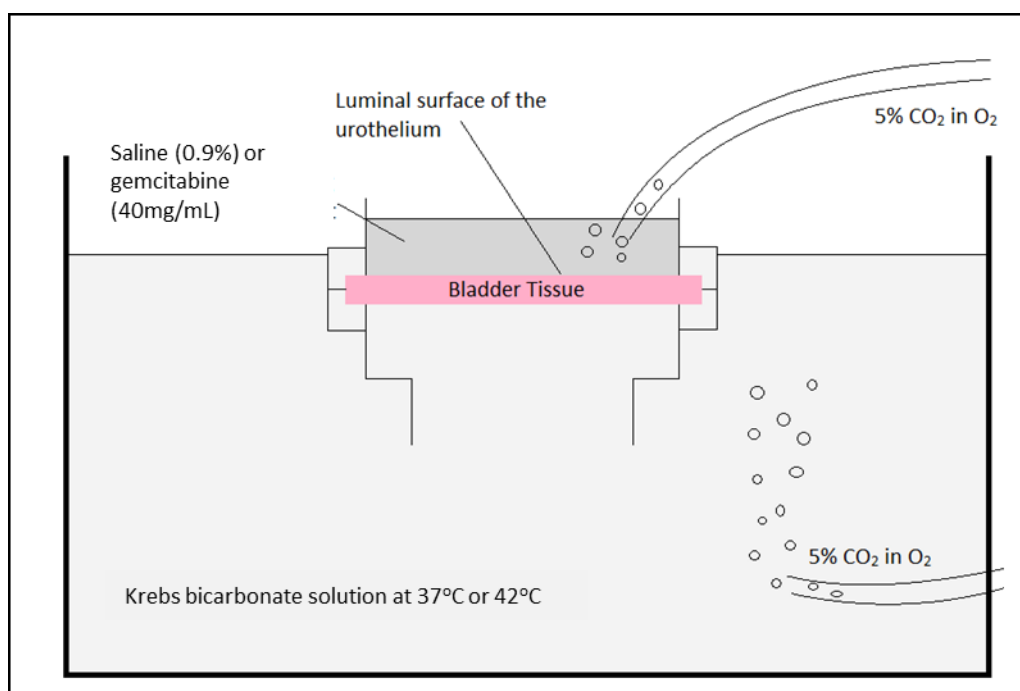
## 4.3 MATERIALS AND METHODS

### ANIMALS

Bladders from mature female pig (sow, >1 year old) were obtained from a local abattoir (Highchester Meats, Beaudesert Queensland) and immediately placed in Krebs-bicarbonate solution (composition in mmol/L: NaCl 118.4,  $\text{NaHCO}_3$  24.9, KCl 4.7,  $\text{CaCl}_2$  1.9,  $\text{MgSO}_4$  1.15,  $\text{KHPO}_4$  1.15, glucose 11.7) at 4°C until use.

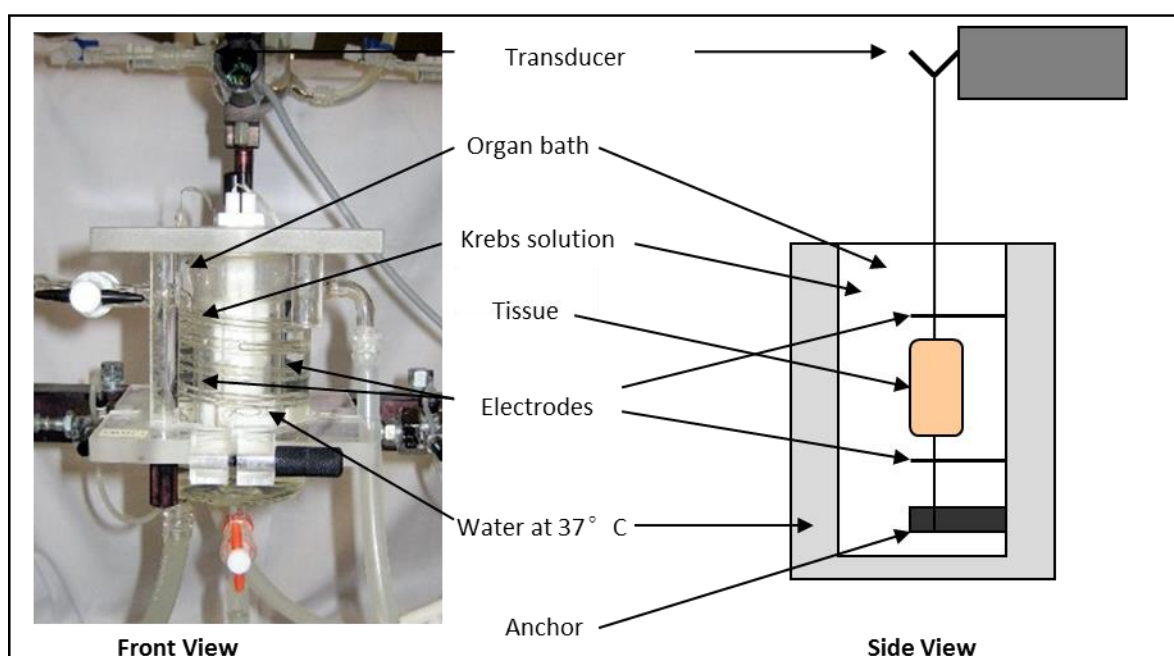
### TISSUE DISSECTION AND EXPERIMENTAL SETUP

The excess adventitial tissue, urethra and ureters were dissected from the bladder. The bladder was opened from the urethra to the dome, and circular sections of intact tissue were cut from the dome of the bladder and mounted in modified Ussing chambers (as described by Smith et al. (2014)) containing Krebs-bicarbonate solution, gassed with 5%  $\text{CO}_2$  in oxygen (**Figure 4.1**). The temperature of the Krebs was either 37°C or 42°C, and 0.9% saline or 40mg/mL gemcitabine (dissolved in 0.9% saline) was applied to the luminal side (urothelial surface) of the bladder for 1 hour. Aluminium foil was placed over the entire unit to regulate temperature.



**Figure 4.1:** Schematic figure of the modified Ussing chamber with a section of intact bladder tissue secured within the apparatus. The tissue was incubated in Krebs-bicarbonate solution on the adventitial side at 37°C or 42°C for 1 hour, with 0.9% saline or 40mg/mL gemcitabine (dissolved in 0.9% saline) applied to the luminal side.

After treatment, the tissues were washed with warm (37°C) Krebs solution and the treated bladder portions were sectioned into strips of intact, denuded detrusor and urothelium/lamina propria tissue, approximately 10mm x 5mm in dimensions. Strips from the respective control and treated tissues were anchored in individual organ baths in Krebs-bicarbonate solution, gassed with 5% CO<sub>2</sub> in oxygen at 37°C (**Figure 4.2**). Tissues were equilibrated for 1 hour under a resting tension of 150mN during which time they were washed with fresh, warmed Krebs-bicarbonate solution every 15 minutes. Tension was measured by an isometric force transducer (ADInstruments, Ltd Australia), recorded by a Powerlab 8/30 recording system (ADInstruments Ltd.) via an Octal Bridge Amp (ADInstruments Ltd.) and analysed using LabChart (version 7.0.3) software (ADInstruments Ltd.).



**Figure 4.2:** Individual organ bath containing a strip of isolated intact, denuded detrusor or urothelium/lamina propria. Tissues were anchored to the bath and connected to an isometric force transducer to record tension developed. The side view is a schematic illustration of the tissue setup, with electrodes placed either side of the tissue.

## SPONTANEOUS ACTIVITY

After equilibration of the tissues, the spontaneous activity of the denuded detrusor and isolated urothelial tissue was investigated. The frequency of phasic contractions was measured after the

equilibration period, recorded as contractions per minute and amplitude measured from the peak to trough of the contraction.

### **FUNCTIONAL STUDIES: PHARMACOLOGICAL AGENTS**

The strips of bladder tissue were contracted to 60mM potassium chloride (KCl), 1mM ATP and a cumulative-concentration curve to carbachol (5.5nM - 165 $\mu$ M). Tissues were washed twice with fresh Krebs-bicarbonate solution and left for at least 15 minutes between agonist additions.

A dose of 16.5 $\mu$ M carbachol (submaximal contraction) was applied to the tissue and contractions allowed to plateau. A cumulative-concentration curve to isoprenaline (48pM - 48 $\mu$ M) was recorded. Responses of each tissue were taken by measuring the change in tension of agonist from baseline (tension prior to addition of agonist). The effect of gemcitabine on the contractility/relaxation response of the detrusor and urothelium/lamina propria was compared to strips of tissue from matched bladders. A comparison of responses of intact bladder tissue with urothelium-denuded tissue was used to examine the inhibitory effect of the urothelium on the detrusor muscle.

### **UROTHELIAL MEDIATOR TRANSMITTER RELEASE**

For mediator release, three strips of isolated urothelium/lamina propria from either control or treated bladders were tied together and anchored to organ baths as described above. Following the equilibration period, 3mL of fresh Krebs solution was added to the bath and left for a period of two minutes. Samples of the Krebs solution were taken immediately after the two-minute period (0min) and 1, 5, 10 and 15 minutes after, with fresh Krebs replacing the volume of collected sample. The Krebs was immediately refreshed and the urothelial strips stretched over a two minute period, evoking approximately 100mN tension. Samples of stimulated (stretched) mediator release were collected immediately, 1, 5, 10 and 15 minutes after the two minute stretch period, with fresh Krebs replacing the volume of collected sample. Samples were stored frozen at -30°C until the levels of ATP, acetylcholine and prostaglandin E<sub>2</sub> from each sample were measured using the respective assay kits, as described in Chapter 2.

## **ELECTRICAL FIELD STIMULATION**

Strips of denuded detrusor from control and gemcitabine treated porcine bladders were dissected and anchored in individual organ baths as described above. The tissue strips were electrically stimulated (20V, 1ms pulse-width, 5s train delivered every 100s) at frequencies of 1, 5, 10 and 20Hz until consistent contractions were obtained (approximately 5-6 contractions/frequency). These stimulations were repeated in the presence of 1 $\mu$ M atropine (competitive muscarinic antagonist) and 10 $\mu$ M  $\alpha,\beta$ -mATP (P2X purinoceptor agonist) to investigate the contributions of acetylcholine and ATP in nerve-mediated contractions.

The addition of 1 $\mu$ M tetrodotoxin (TTX) to the strips of denuded detrusor during preliminary experiments confirmed the neural selectivity of contraction of the tissue by abolishing the evoked responses of EFS.

## **HISTOLOGY AND IMMUNOHISTOCHEMISTRY**

After luminal treatment of gemcitabine or saline at 37°C or 42°C, tissues were dissected into thin (~1-2mm wide) strips of intact bladder and placed in neutral buffered formalin (10%) at 4°C for 24 hours. Preparation into wax blocks, processing and staining with haematoxylin and eosin as per the protocol in Chapter 2 was performed to visualise possible urothelial damage.

Sections of tissues were prepared into wax blocks as per the protocol in Chapter 2, and given to Professor Gordon Wright, who kindly performed immunohistochemical staining using commercially available human cytokeratin-7 and -20 antibodies at the Pathology Department of the Gold Coast University Hospital.

## **STATISTICAL ANALYSIS**

Results were expressed as mean  $\pm$  standard error of the mean (SEM). Data were analysed using an ANOVA with Dunnett or Bonferroni post-test using Graphpad Instat (version 3.06, San Diego, CA), and whole curve analyses were performed with a multiple comparisons F-test using GraphPad Prism (version 5.0). Significance levels were defined as \* $p$ <0.05, \*\* $p$ <0.01 and \*\*\* $p$ <0.001.

## 4.4 RESULTS

Luminal treatment of the bladder involves direct contact between the urothelium and cytotoxic drugs. The damaging effect of gemcitabine alone and in combination with hyperthermia on the urothelium was investigated with H&E staining of intact sections of porcine bladders treated with gemcitabine (40mg/mL) at 37°C and 42°C, and compared to matched controls treated with saline (0.9%) (**Figure 4.4**). Typical histological features of a porcine bladder including an intact, folded urothelium and lamina propria layer sitting atop a smooth muscle layer were apparent in the control tissues incubated at both temperatures (**Figure 4.4 A&B, E&F**). The urothelium of the gemcitabine treated tissues appeared to have a less folded appearance, minor damage and thinning in some sections with comparison to the control tissues (**Figure 4.4C&D, G&H**). However, while the thickness of the urothelium in gemcitabine and/or hyperthermia pre-treated tissues was less than control; this difference was not significant (**Figure 4.3**).

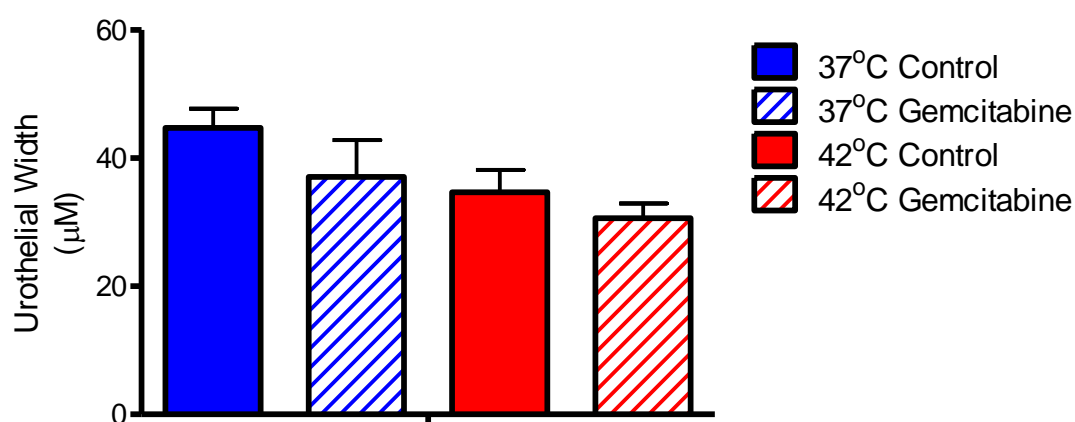


Figure 4.3: Urothelial thickness (μM) in control and gemcitabine treated tissues at 37°C and 42°C. Data represented as mean ± SEM (taken from the average of >10 measurements from n=3 bladders)



## Pre-Treatment

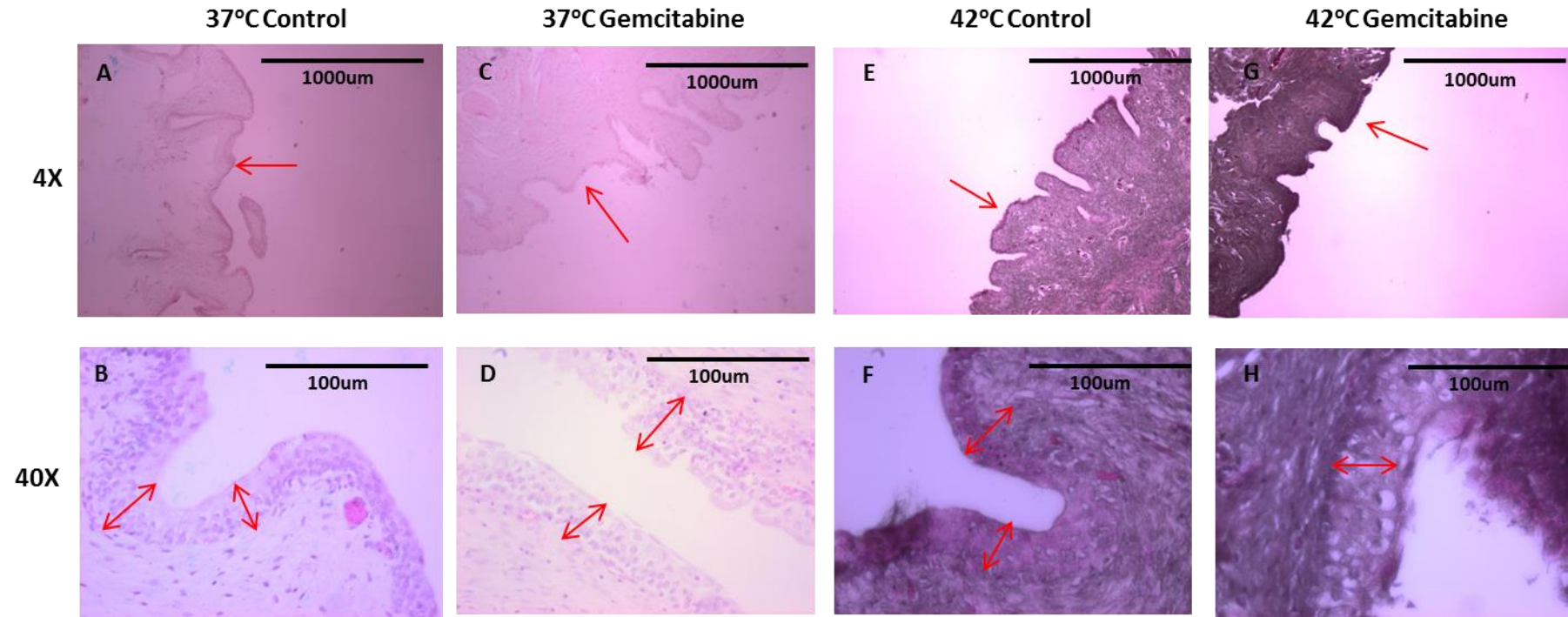


Figure 2: Histological sections (H&E stain) of bladder wall from bladders pre-incubated with saline (A&B) or gemcitabine (C&D) at 37°C or pre-incubated at 42°C with saline (E&F) or gemcitabine (G&H). Red arrows indicate the width of the urothelium.

The typical features of porcine bladders were similarly apparent in the cytokeratin 7 immunohistochemically stained sections (**Figure 4.5**). Cytokeratin 7 is a protein expressed by all cells of the urothelium – basal, intermediate and umbrella cells. The luminal side of the urothelium in control treated tissues (**Figure 4.5 A&B, E&F**) is smooth and folded, while there is evident damage of the urothelial barrier and a less folded appearance in gemcitabine pre-treated tissues (**Figure 4.5 C&D, G&H**). As previously described, cytokeratin 20 is expressed in the urothelium, usually restricted to the umbrella and occasionally intermediate cell layers as it is associated with differentiation (Romih et al., 1998, Romih et al., 2005, Moll et al., 1992, Moll, 1991). The expression of this protein was in abundance (indicated by red arrows) in the control tissues incubated at 37°C (**Figure 4.6 A&B**). In the gemcitabine treated tissues at 37°C (**Figure 4.6 C&D**), staining for cytokeratin 20 was sporadic, while both the control (**Figure 4.6 E&F**) and gemcitabine pre-treated tissues incubated at 42°C (**Figure 4.6 G&H**) had very few stained cells. This is indicative of sloughing of the luminal layers of the urothelium by gemcitabine and hyperthermia treatment.

## Pre-Treatment

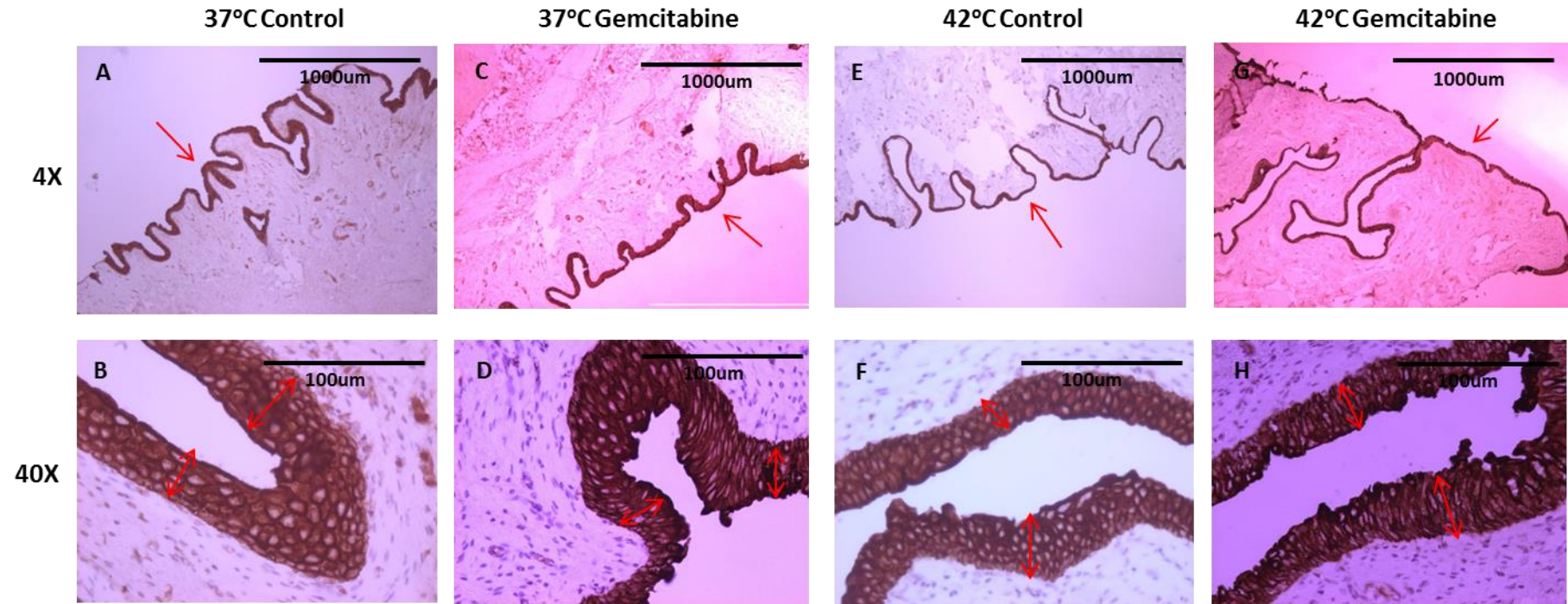


Figure 4.5: Cytokeratin 7 stained sections of bladder wall from bladders pre-incubated with saline (A&B) or gemcitabine (C&D) at 37°C or pre-incubated at 42°C with saline (E&F) or gemcitabine (G&H). Red arrows indicate the width of the urothelium.

# Pre-Treatment

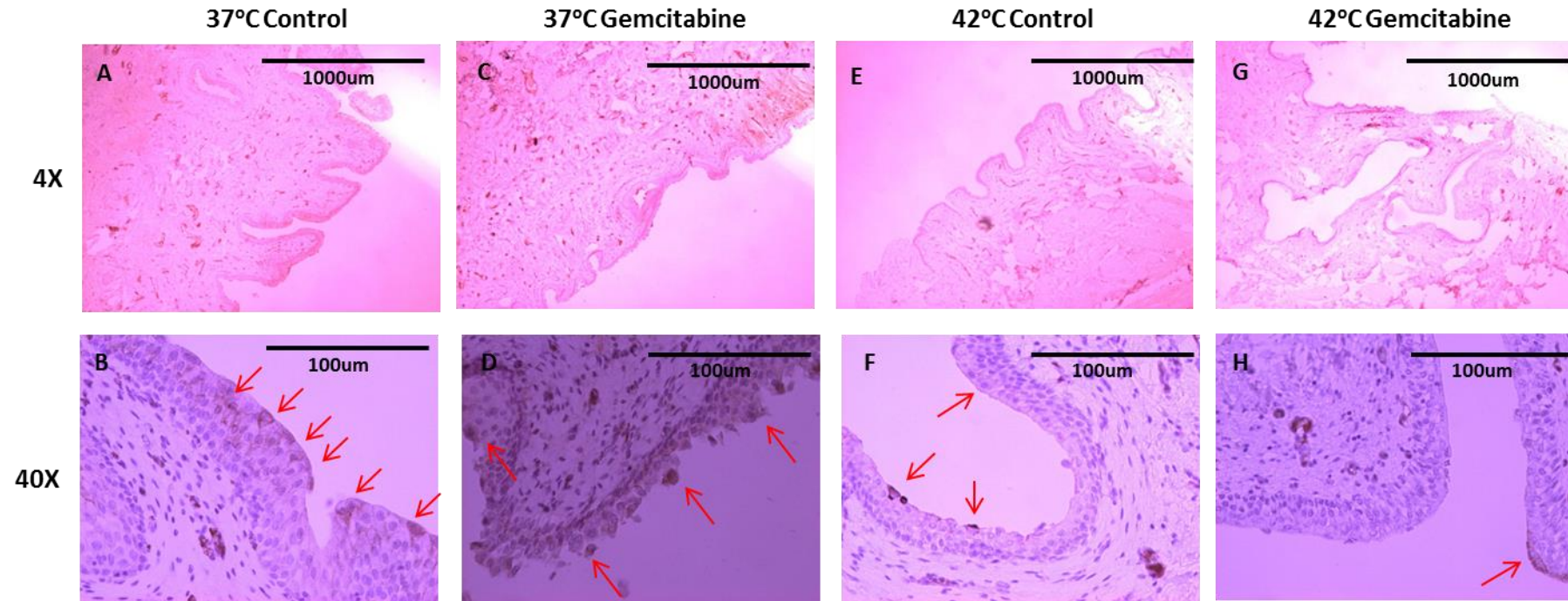


Figure 4.6: Cytokeratin 20 stained sections of bladder wall from bladders pre-incubated with saline (A&B) or gemcitabine (C&D) at 37°C or pre-incubated at 42°C with saline (E&F) or gemcitabine (G&H). The cells stained for cytokeratin 20 (maroon in colour) are indicated by red arrows.

### **UROTHELIAL MEDIATOR RELEASE: INCUBATION AT 37°C**

The effect of luminal gemcitabine on basal and stretched urothelial mediator release was measured from strips of isolated urothelium/lamina propria. In control pre-incubated tissues, no difference was observed between release of mediators ATP, acetylcholine or prostaglandin E<sub>2</sub> between basal or stretched states. However, luminal gemcitabine pre-treatment decreased ATP release both basally and with stretch, significantly reduced by approximately 80% after 1 and 15 minutes, and reduced to approximately 10% of control response after 1, 5 and 10 minutes after stretch (**Figure 4.7 A&B**).

The bath concentration of acetylcholine increased in a time dependent manner under basal conditions in tissues from control and gemcitabine pre-treated bladders (**Figure 4.7C**). Stretch did not enhance acetylcholine release and luminal gemcitabine pre-treatment also had no effect on release (**Figure 4.7 C&D**).

Prostaglandin E<sub>2</sub> release similarly increased in a time-dependent manner (**Figure 4.7 E&F**). This was also seen in gemcitabine pre-treated tissues. Luminal pre-treatment with gemcitabine significantly enhanced subsequent prostaglandin release compared to matched control tissues, by a factor of 3 in both the basal and stretched conditions at each time point measured.

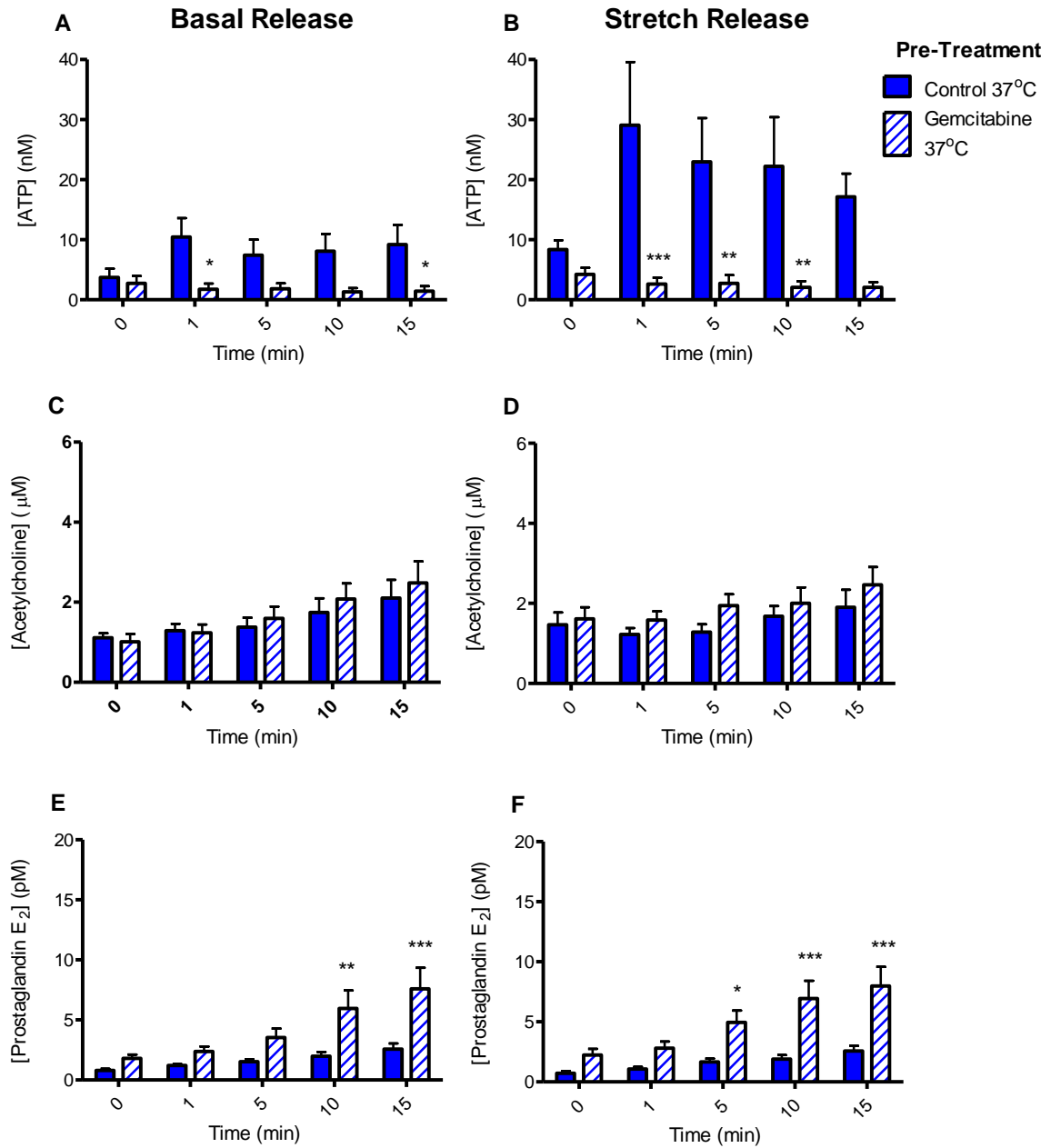


Figure 4.7: Basal and stretch induced ATP (A&B), acetylcholine (C&D) and prostaglandin E<sub>2</sub> (E&F) from isolated strips of urothelium/lamina propria from porcine bladders pre-treated with luminal saline (0.9%) or gemcitabine (40mg/mL) at 37°C for 1 hour. Data is represented as mean  $\pm$  SEM ( $n \geq 4$ ), analysed by an one-way ANOVA with Bonferroni post-test (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs control)

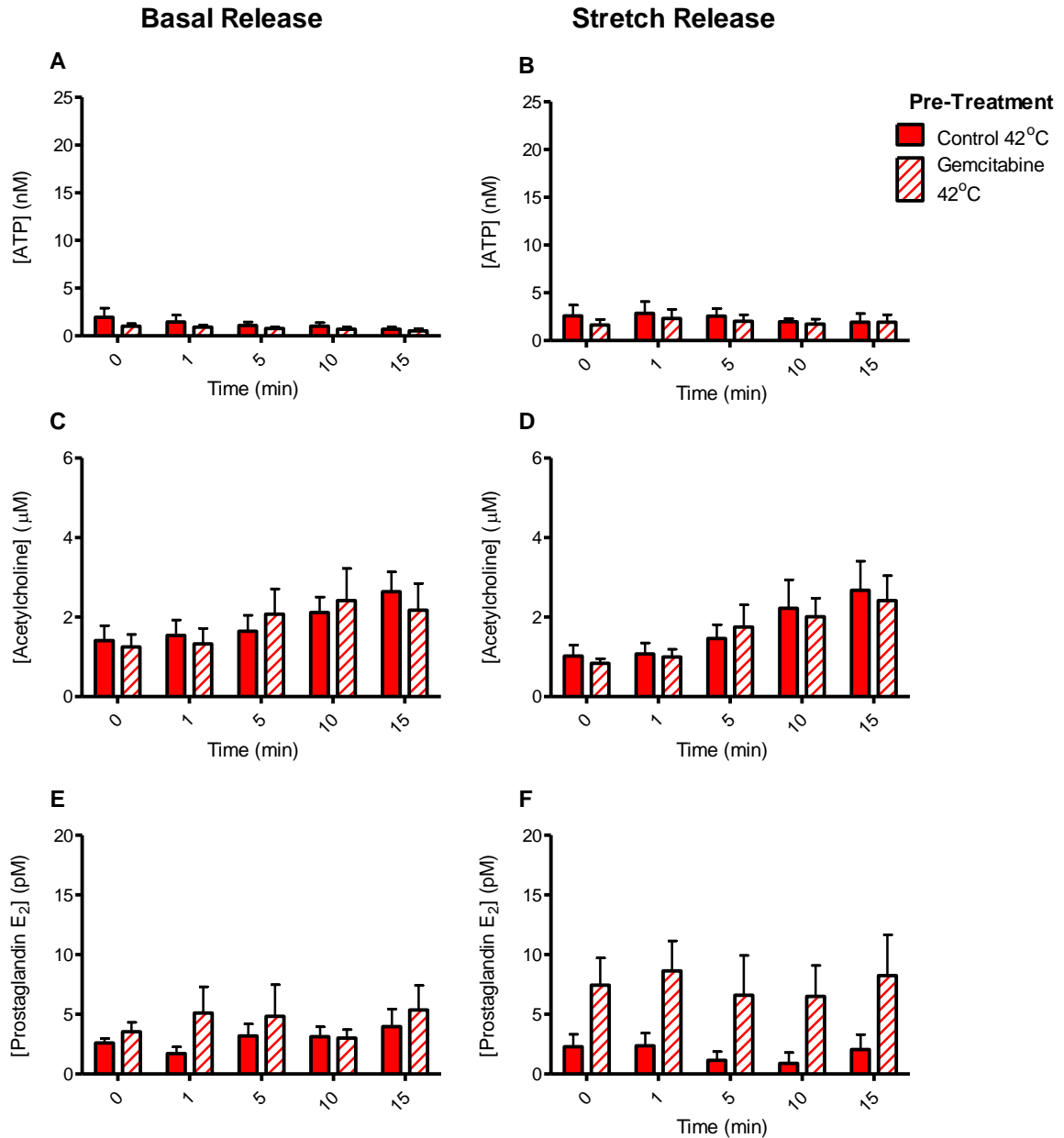


## UROTHELIAL MEDIATOR RELEASE: INCUBATION AT 42°C

The effect of incubation of tissues at 42°C and in combination luminal gemcitabine treatment was examined. With comparison to control tissues incubated at 37°C, the release of ATP both basally and with stretch from control tissues incubated at 42°C was reduced by >80% across all time points after 1 minute (**Figure 4.7 A&B, 4.8 A&B**). With hyperthermia pre-treatment, there was no difference between basal and stretch-induced release of ATP, nor did gemcitabine alter the release of ATP with comparison to the matched controls.

As observed following pre-incubation at 37°C, release of acetylcholine at basal conditions increased in a time dependent manner in both control and gemcitabine pre-treated tissues at 42°C (**Figure 4.8 C&D**). No change was observed in the release of acetylcholine from tissues pre-incubated at 42°C with comparison to the 37°C, nor did luminal gemcitabine pre-treatment have any effect on acetylcholine release with comparison to control tissues pre-incubated at 42°C.

Pre-incubation at 42°C enhanced basal release of prostaglandin E<sub>2</sub> from control tissues with comparison to the 37°C incubation (**Figure 4.8 E&F**). Luminal gemcitabine pre-treatment at 42°C enhanced release of prostaglandin E<sub>2</sub> from control tissues, however this was not significant as some samples were below the level of detection of the assay.



**Figure 4.8:** Basal and stretch induced ATP (A&B), acetylcholine (C&D) and prostaglandin E<sub>2</sub> (E&F) from isolated strips of urothelium/lamina propria from porcine bladders, pre-treated with luminal saline (0.9%) or gemcitabine (40mg/mL) at 42°C for 1 hour. Data is represented as mean  $\pm$  SEM ( $n \geq 4$ ), analysed by an one-way ANOVA with Bonferroni post-test (\* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs control)



## SPONTANEOUS ACTIVITY OF THE BLADDER

Spontaneous contractions of denuded detrusor strips and urothelium/lamina propria tissue strips after one hour equilibration were examined. The urothelium/lamina propria contracted spontaneously, as seen in **Figure 4.9**. Isolated strips of denuded detrusor also displayed spontaneous contraction, although in tissues treated at 42°C, these contractions were only discernible in 3 (out of 8) tissues. Both amplitude and frequency of contractions evoked by denuded detrusor strips were significantly depressed with comparison to the matched isolated urothelial strips (**Figure 4.10 A&B**). Treatment with gemcitabine or increased pre-incubation temperature did not change the spontaneous contraction of either denuded detrusor or urothelium/lamina propria

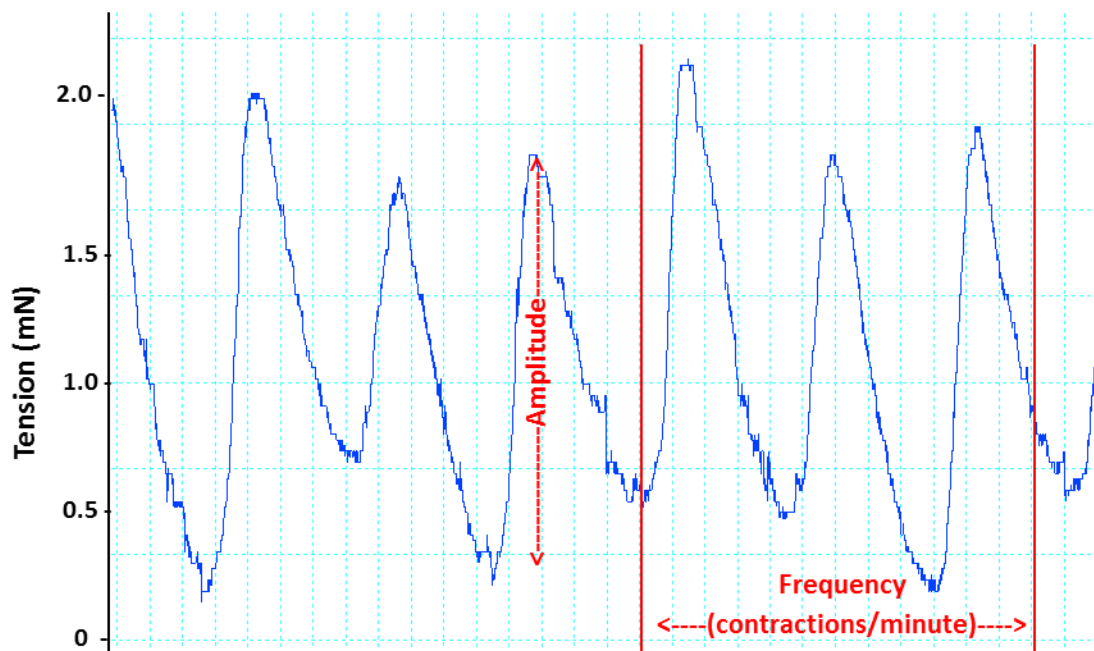


Figure 4.9: Representative trace of spontaneous activity from strips of isolated porcine urothelium/lamina propria

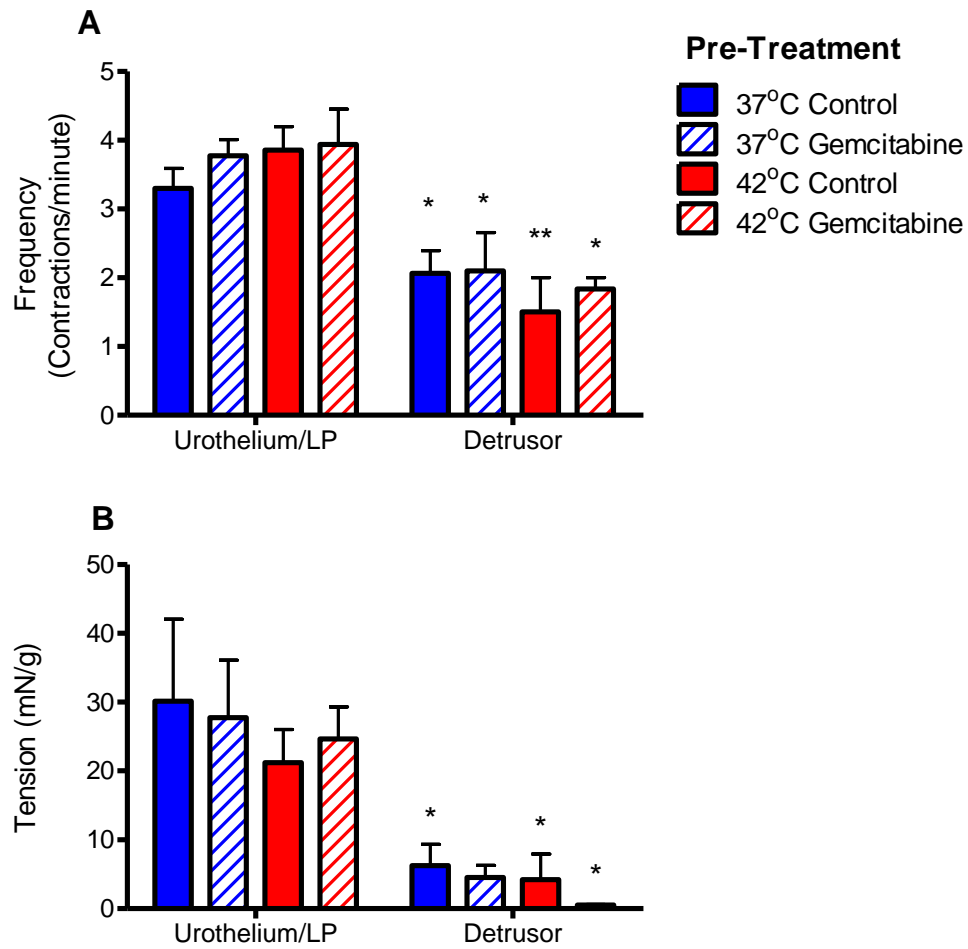


Figure 4.10: Frequency (A) and amplitude (B) of spontaneous contractions from isolated strips of porcine urothelial and denuded detrusor tissue from bladders pre-treated with luminal saline (0.9%) or gemcitabine (40mg/mL) for 1 hour, incubated at 37°C or 42°C. Data represented as mean  $\pm$  SEM ( $n \geq 3$ ), contraction normalised by tissue weight, analysed with an unpaired two-tailed t-test (\* $p < 0.05$ , \*\* $p < 0.01$  vs matched urothelial strips)

## CONTRACTILITY OF TISSUE STRIPS

The effect of pre-incubation gemcitabine and increased temperature on tissue contractility was examined in urothelium/lamina propria, denuded detrusor and intact bladder strips. Contraction to KCl (60mM) and ATP (1mM) was investigated first.

In urothelial and intact bladder strips, neither gemcitabine nor hyperthermia pre-treatment had any effect on either responses to KCl (**Figure 4.11 A&C**) or ATP (**Figure 4.12 A&C**). For the denuded detrusor strips, combined gemcitabine and hyperthermia pre-treatment reduced contraction to KCl by 80% (**Figure 4.11B**), while contraction to ATP was reduced by approximately 75% and 90% respectively by hyperthermia pre-treatment alone and in combination with gemcitabine (**Figure 4.12B**).

Also, the inhibitory effect of the urothelium on the detrusor muscle was observed in the control intact tissues; inhibiting contraction to KCl by 65% and 62% at pre-incubation temperatures 37°C and 42°C respectively, while this effect was abolished with gemcitabine pre-treatment (**Figure 4.11C**). This inhibitory effect of the urothelium on intact tissues was observed in contraction to ATP of control tissues, reducing by 60%. This was effect was not observed in gemcitabine pre-treated tissues, nor the 42°C pre-incubated control (**Figure 4.12C**).

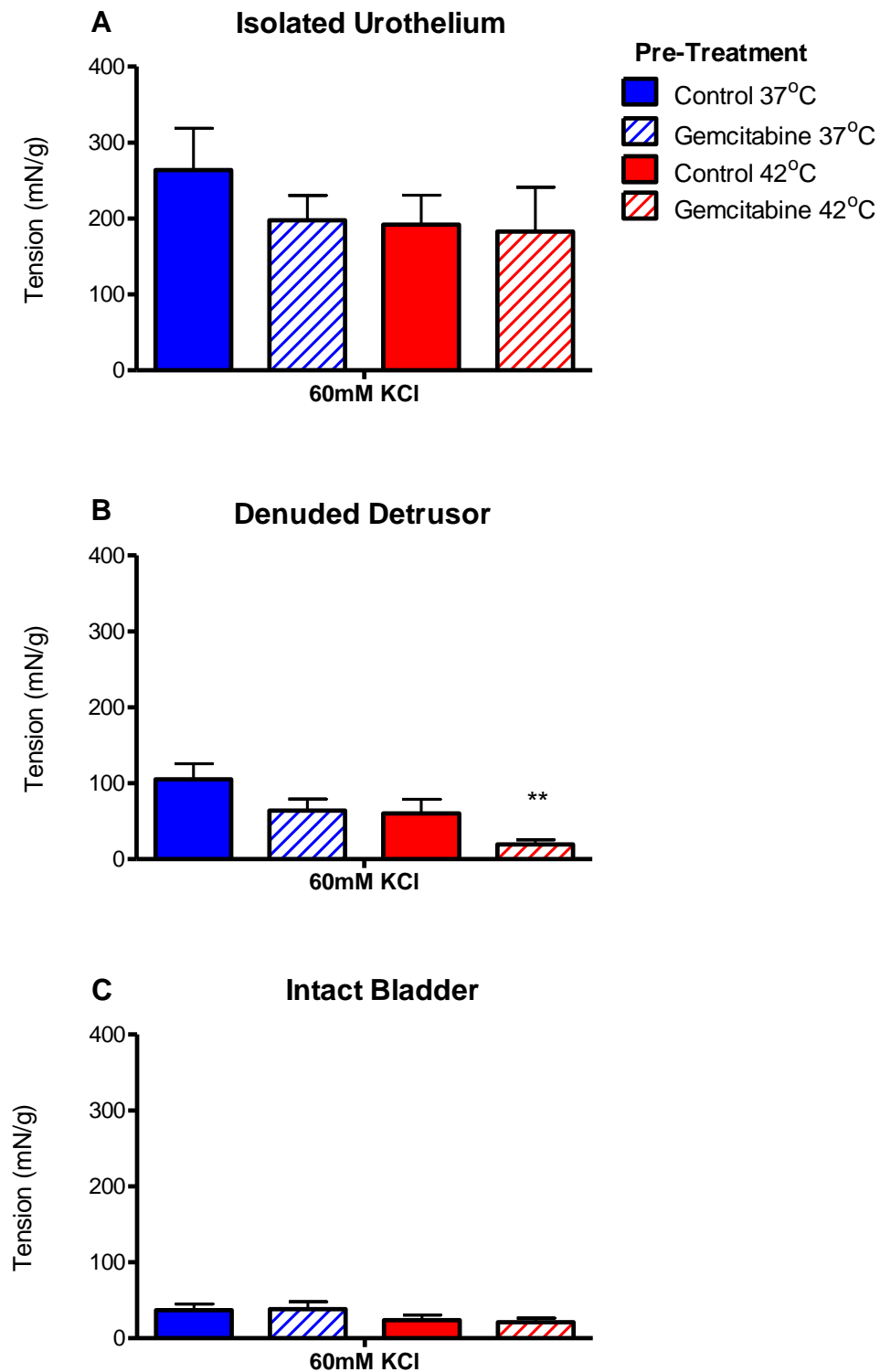


Figure 4.11: Effect of 1hr luminal pre-treatment with saline (0.9% - control) or gemcitabine (40mg/mL) at either 37°C or 42°C on the contractility of isolated urothelial (A), denuded detrusor (B) and intact (C) porcine tissue to 60mM KCl. Data represented as mean  $\pm$  SEM ( $n \geq 7$ ), contraction normalised by tissue weight, analysed by a one-way ANOVA with Dunnett post-test (\*\*  $p < 0.01$  vs 37°C control).

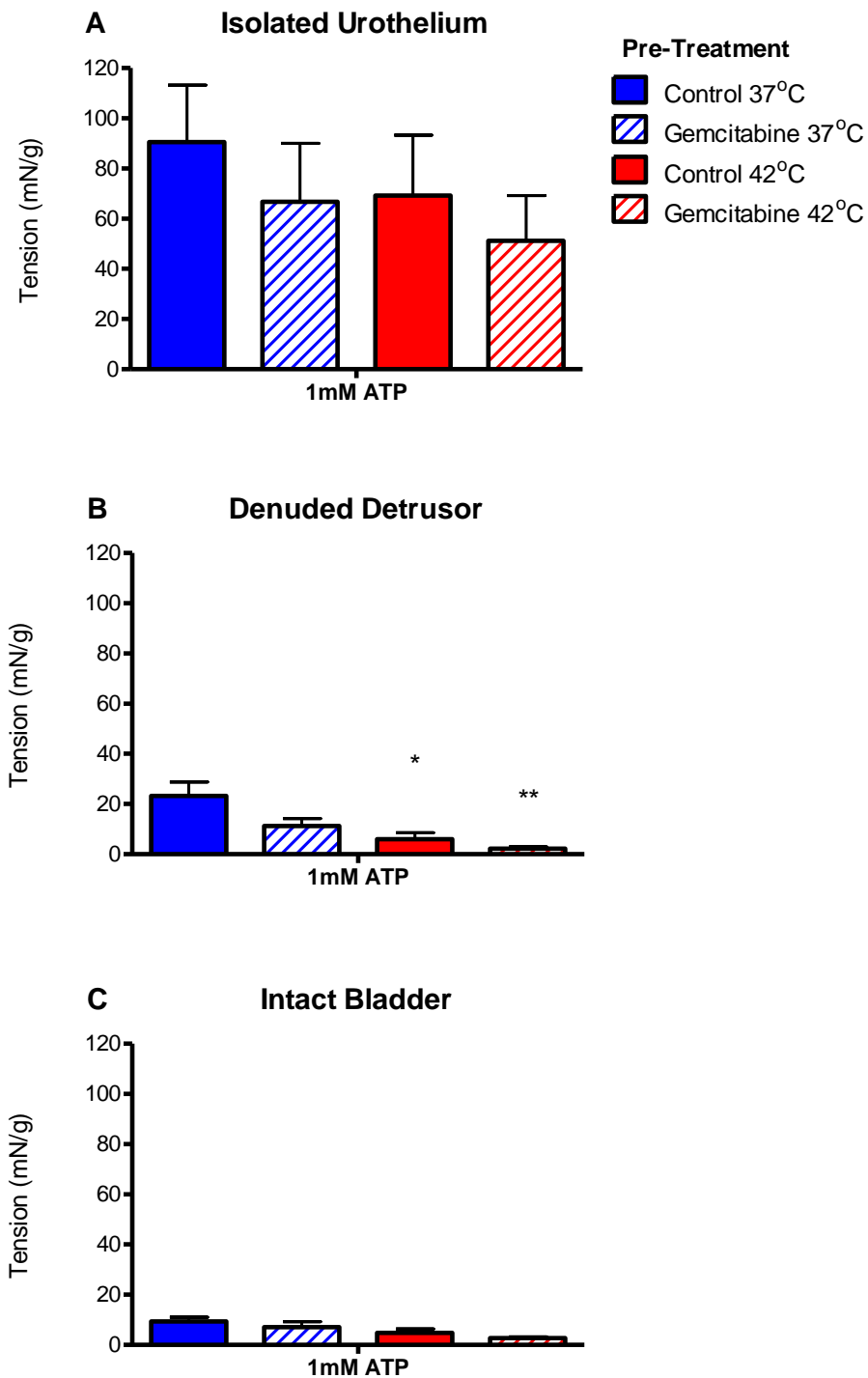


Figure 4.12: Effect of 1hr luminal pre-treatment with saline (0.9% - control) or gemcitabine (40mg/mL) at either 37°C or 42°C on the contractility of isolated urothelial (A), denuded detrusor (B) and intact (C) porcine tissue to 1mM ATP. Data represented as mean  $\pm$  SEM ( $n \geq 7$ ), contraction normalised by tissue weight, analysed by a one-way ANOVA with Dunnett post-test (\*  $p < 0.05$ , \*\*  $p < 0.01$  vs 37°C control).

## RESPONSE TO PHARMACOLOGICAL AGENTS

Next, the effect of pre-treatment with gemcitabine and/or increased temperature on response of strips of porcine tissue to the muscarinic agonist carbachol and  $\beta$ -adrenergic agonist isoprenaline was investigated.

### CONTRACTION RESPONSE TO CARBACHOL

**Figure 4.13** depicts representative contractions of a denuded detrusor muscle strip to cumulative concentrations of carbachol. In all tissues, contractions to cumulative additions of carbachol were depressed across the curve by gemcitabine and/or hyperthermia pre-treatment (**Figure 4.14 A-C**). However, when presented as % contraction to the maximum, the potency of carbachol was similar in all tissue groups (**Figure 4.14 D-F**).

The maximal contractions to carbachol in the isolated urothelium were depressed in the gemcitabine pre-treated tissues, being reduced by 42% and 48% in the 37°C and 42°C incubation groups respectively (**Figure 4.14A, Table 4.1**).

Similarly, with comparison to the 37°C control response of the denuded detrusor, the maximal contraction to carbachol was reduced 42% and 80% by gemcitabine pre-treatment at 37°C and 42°C respectively (**Figure 4.14B, Table 4.1**).

Finally, contraction to carbachol was measured in strips of intact bladder tissue. The inhibitory effect of the urothelium on detrusor maximal contraction to carbachol was observed in control tissues pre-incubated at both 37°C (449±106mN/g denuded detrusor, 224±53mN/g intact,  $p=0.04$ ) and 42°C (308±103mN/g denuded detrusor, 64±14mN/g intact,  $p=0.03$ ), while this inhibitory effect was abolished in gemcitabine pre-treated tissues. The maximal contraction of the intact tissue to carbachol was significantly reduced from control tissues in the hyperthermia pre-treated group, by 75% (control 42°C) and 71% (gemcitabine 42°C) respectively (**Figure 4.14C, Table 4.1**).

Although pre-incubation with gemcitabine, increased temperature, or combined gemcitabine and hyperthermia reduced the maximal contractions to carbachol; there was no change in the pEC<sub>50</sub> values of carbachol in each tissue type between treatment groups (**Table 4.1**).

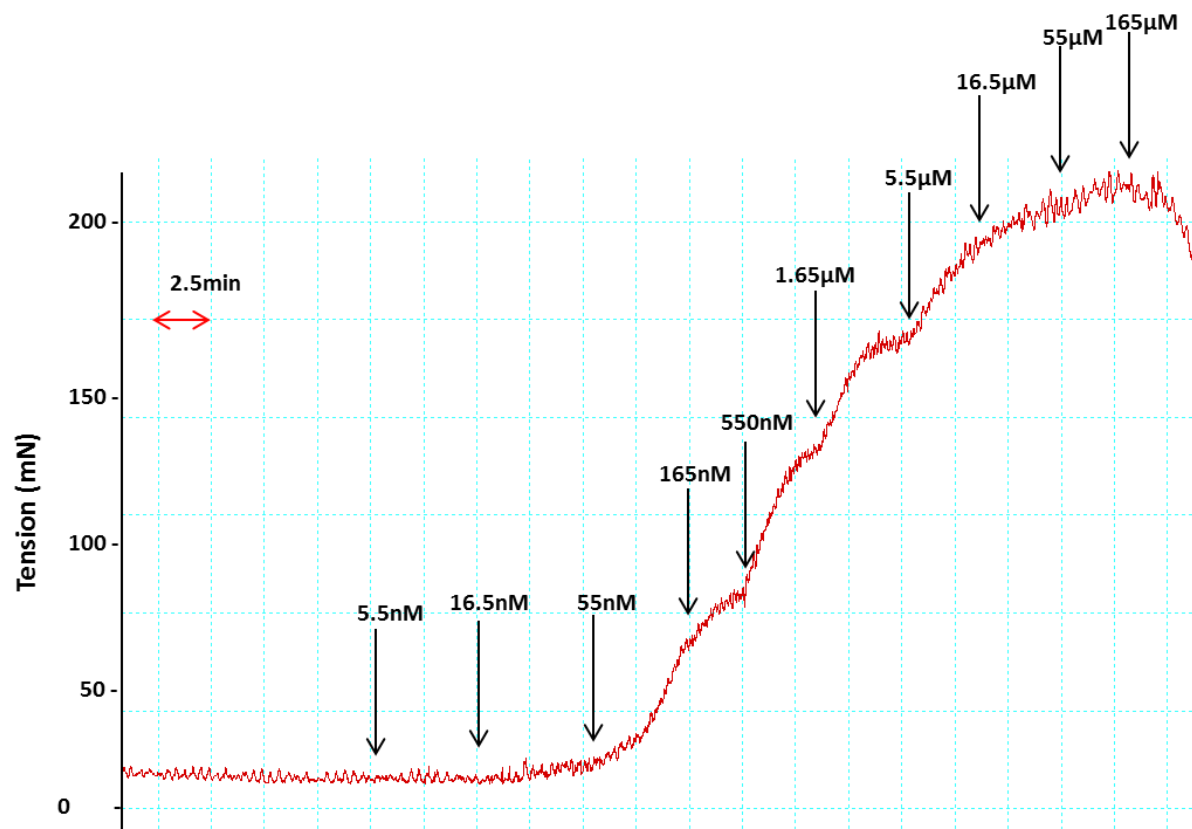
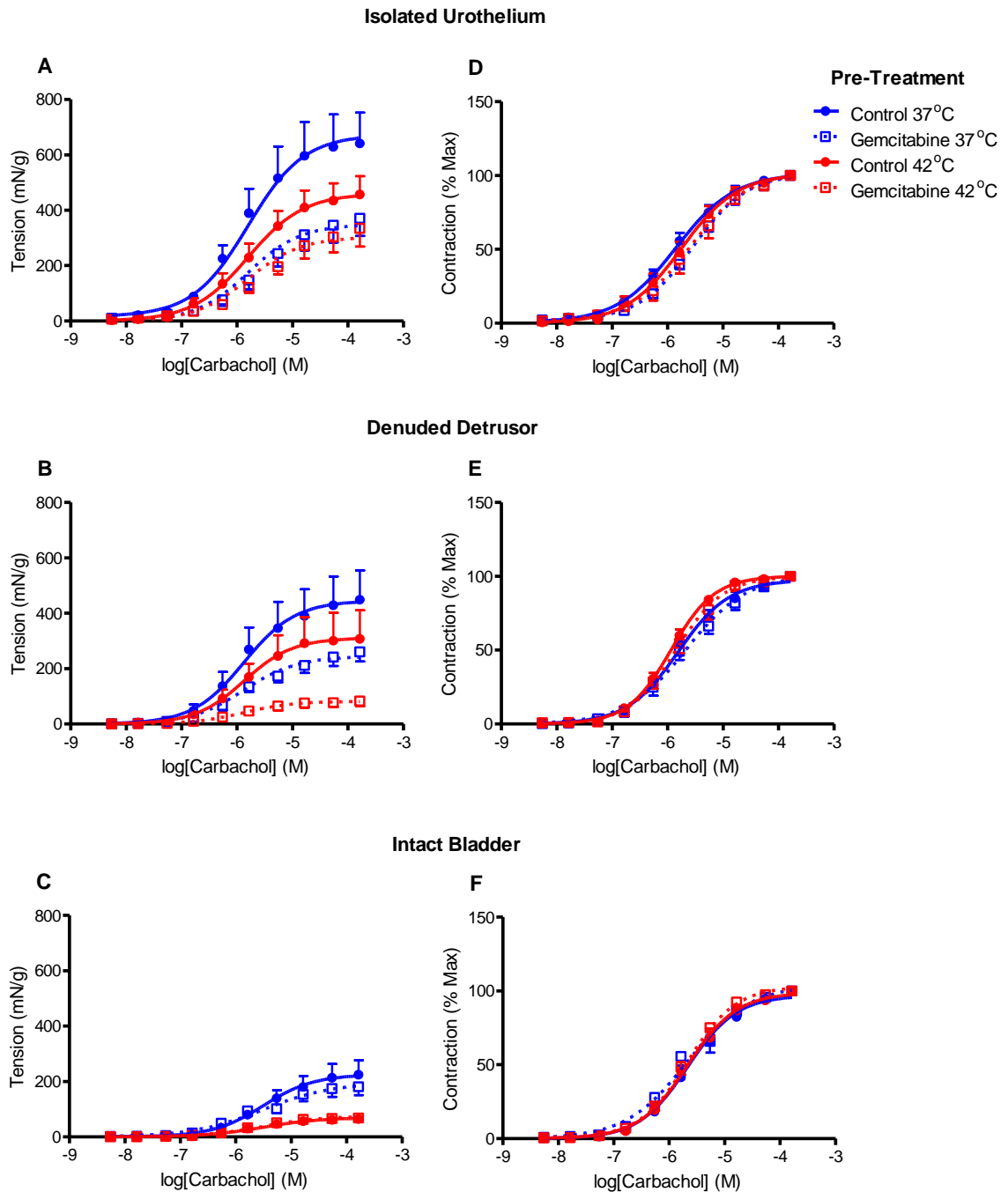


Figure 4.13: Representative trace of denuded porcine detrusor response to cumulative concentrations of carbachol.



**Figure 4.14: Effect of 1hr luminal pre-treatment with saline (0.9% control) or gemcitabine (40mg/mL) at 37°C or 42°C on the contraction of isolated urothelium (A&D), denuded detrusor (B&E) and intact (C&F) porcine tissue to cumulative carbachol concentrations (A-C) and contraction represented as a % of the maximum of the respective control/treatment groups (D-F). Data represented as mean  $\pm$  SEM ( $n \geq 7$ ), contraction normalised by tissue weight.**



Table 4.1: Effect of 1hr luminal pre-treatment with saline (0.9% - control) or gemcitabine (40mg/mL) incubation at 37°C or 42°C on the pEC50 (SEM) and EC50 (95% CI) values for carbachol in isolated porcine urothelial, denuded detrusor and intact tissue strips. Data analysed with a multiple comparison F test (n≥7) and one-way ANOVA using Dunnett post-test (\*p<0.05 with comparison to 37°C Control)

	37°C Control	37°C Gemcitabine	42°C Control	42°C Gemcitabine
<b>Isolated Urothelial/Lamina Propria Tissue</b>				
<b>pEC50 (± SEM)</b>	6.0 ± 0.2	5.5 ± 0.2	5.8 ± 0.2	5.4 ± 0.2
<b>EC50 (95% CI)</b>	1.1 (0.5–2.9) µM	3.0 (1.2–7.8) µM	1.6 (0.7–3.7) µM	3.9 (1.4–10.9) µM
<b>Maximum Response (mN/g)</b>	641.6 ± 111.6	371.2 ± 64.4*	456.8 ± 66.8	335 ± 66
<b>Denuded Detrusor Tissue</b>				
<b>pEC50 (± SEM)</b>	5.9 ± 0.3	5.7 ± 0.2	5.9 ± 0.3	5.9 ± 0.2
<b>EC50 (95% CI)</b>	1.2 (0.4–4) µM	2.0 (0.9–4.4) µM	1.4 (0.3–5.5) µM	1.3 (0.4–3.7) µM
<b>Maximum Response (mN/g)</b>	449 ± 105.8	260.7 ± 34.8	307.6 ± 102.9	82.8 ± 19.3**
<b>Intact Tissue</b>				
<b>pEC50 (± SEM)</b>	5.4 ± 0.2	5.6 ± 0.2	5.7 ± 0.2	5.7 ± 0.1
<b>EC50 (95% CI)</b>	3.6 (1.2–10.6) µM	2.8 (0.9–8.6) µM	2.0 (0.8–4.8) µM	1.9 (1.1–3.3) µM
<b>Maximum Response (mN/g)</b>	224.5 ± 52.8	180.9 ± 30.5	64.4 ± 14.4*	68.2 ± 9*

## RELAXATION RESPONSE TO ISOPRENALINE

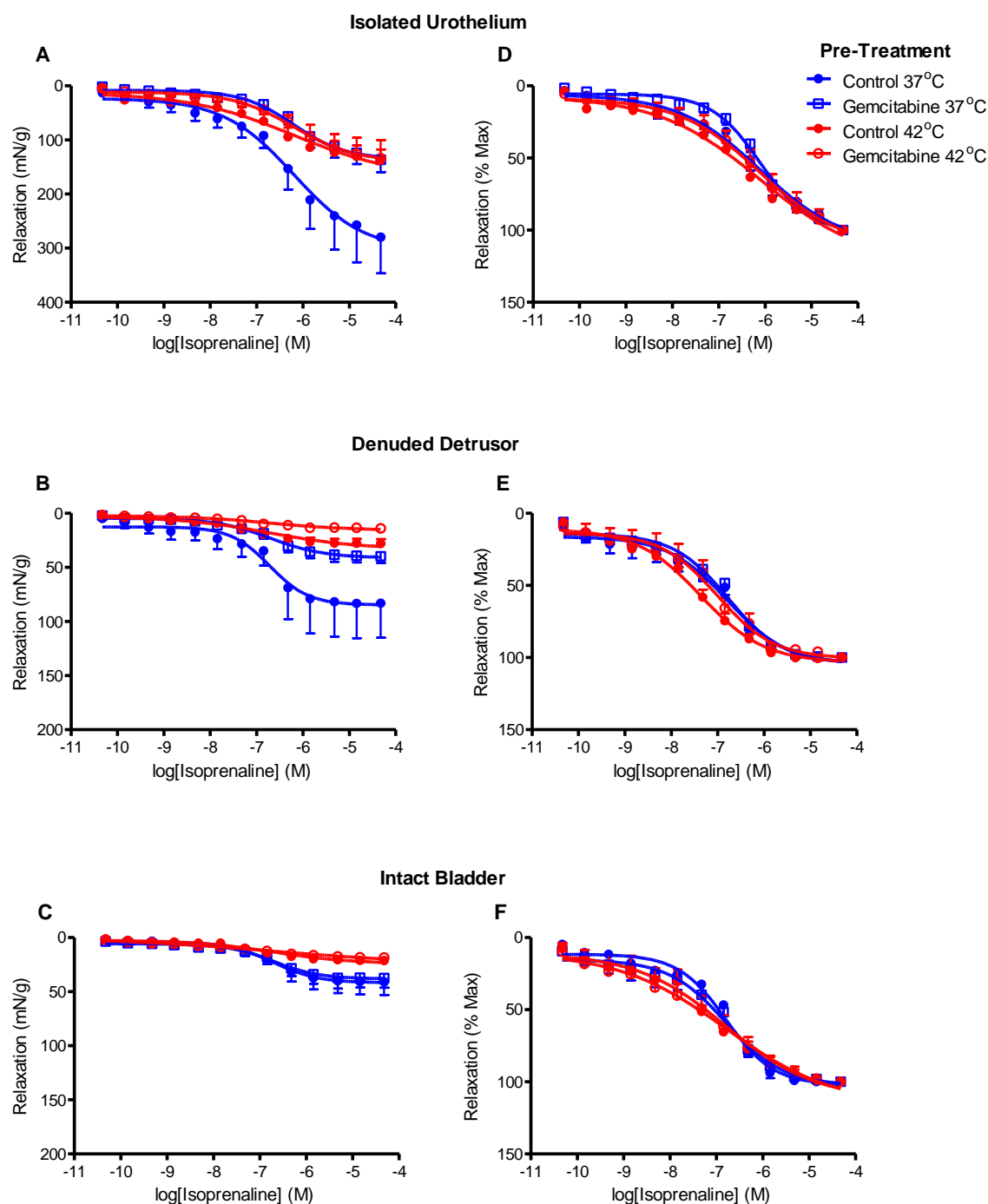
After pre-contraction of the tissues with carbachol, the addition of isoprenaline caused a concentration-dependent relaxation. In strips of urothelium/lamina propria, denuded detrusor and intact tissue, relaxation to cumulative additions of isoprenaline were depressed by gemcitabine and/or hyperthermia pre-treatment (**Figure 4.15 A-C**). However, when presented as % contraction to the maximum, there was no change between treatment groups in each of the tissue types (**Figure 4.15 D-F**).

The maximal relaxation to isoprenaline in the isolated urothelium was depressed by over 50% in all pre-treatment groups (**Figure 4.15A**). However, this was only significant in the 37°C gemcitabine pre-treated group (**Table 4.2**).

In the denuded detrusor tissue, only the gemcitabine and combined hyperthermia pre-treatment group significantly reduced the maximal response to isoprenaline by >80% (**Figure 4.15B, Table 4.2**).

While the maximal response to isoprenaline was reduced from control in strips of intact bladder tissue pre-treated with gemcitabine and/or hyperthermia, this effect was not significant (**Figure 4.15C, Table 4.2**). The presence of the urothelium did not affect relaxation to isoprenaline.

Although pre-incubation with gemcitabine and/or hyperthermia reduced the maximal relaxation response to isoprenaline; there was no difference in the potency of this response as evidenced by the unchanged pEC<sub>50</sub> values of isoprenaline in each tissue type between treatment groups (**Table 4.2**).



**Figure 4.15: Effect of 1hr luminal pre-treatment with saline (0.9% - control) or gemcitabine (40mg/mL) incubation at 37°C or 42°C on the relaxation response of isolated urothelial (A&D), denuded detrusor (B&E) and intact (C&F) porcine tissue to cumulative isoprenaline concentrations (A-C) and relaxation represented as a % of the maximum of the respective control/treatment groups (D-F). Data represented as mean  $\pm$  SEM (n $\geq$ 6), relaxation normalised by tissue weight.**

Table 4.2: Effect of 1hr luminal pre-treatment with saline (0.9% - control) or gemcitabine (40mg/mL) at 37°C or 42°C on the pEC50 (SEM) and EC50 (95% CI) values for isoprenaline in isolated porcine urothelial/lamina propria, denuded detrusor and intact tissue strips. Data analysed with a multiple comparison F test (n≥6) and one-way ANOVA using Dunnett post-test (\*p<0.05 with comparison to 37°C Control)

	37°C Control	37°C Gemcitabine	42°C Control	42°C Gemcitabine
<b>Isolated Urothelial/Lamina Propria Tissue</b>				
<b>pEC50 (± SEM)</b>	6.3 ± 0.4	6.1 ± 0.3	6.6 ± 0.4	6.1 ± 0.4
<b>EC50 (95% CI)</b>	0.5 (0.1–4.0) µM	0.9 (0.3–3.0) µM	0.3 (0.1–1.3) µM	0.7 (0.1–4.3) µM
<b>Maximum Response (mN/g)</b>	279.9 ± 66.8	136.7 ± 23.3*	137.3 ± 19.5	133.8 ± 33.1
<b>Denuded Detrusor Tissue</b>				
<b>pEC50 (± SEM)</b>	6.7 ± 0.4	6.7 ± 0.2	7.2 ± 0.2	7.1 ± 0.4
<b>EC50 (95% CI)</b>	0.2(0.03–1.2) µM	0.2 (0.1–0.5) µM	0.03 (0.02–0.5) µM	0.08 (0.01–0.6) µM
<b>Maximum Response (mN/g)</b>	83.2 ± 32.1	39.9 ± 6.3	28.1 ± 3.9	14.1 ± 2.4*
<b>Intact Tissue</b>				
<b>pEC50 (± SEM)</b>	6.7 ± 0.3	6.7 ± 0.3	7.1 ± 0.2	7.3 ± 0.8
<b>EC50 (95% CI)</b>	0.2 (0.05–0.9) µM	0.2 (0.05–0.8) µM	0.07 (0.03–0.2) µM	0.06 (0.001–1.6) µM
<b>Maximum Response (mN/g)</b>	41.3 ± 12	37.5 ± 9	21.4 ± 3.2	18.6 ± 3.2

## CONTRACTION TO ELECTRICAL FIELD STIMULATION

Electrical field stimulation was used to investigate the effect of pre-treatment with gemcitabine and hyperthermia on the efferent nerve mediated contraction in porcine bladders. **Figure 4.16A** depicts a trace of control (37°C pre-treatment) denuded detrusor contraction to EFS. The contractile response increased with increasing frequency of stimulation, and was knocked down with the addition of atropine (1µM) and α,β-mATP (10µM).

The addition of the muscarinic antagonist atropine significantly reduced contraction of the denuded detrusor at high frequencies by 74% (10Hz) and 85% (20Hz) respectively (**Figure 4.16B, Table 4.3**). Desensitization of the P2X purinergic receptors with α,β-mATP reduced contraction by 90-84% from initial values at low stimulation frequencies (1 & 5Hz) as shown in **Table 4.3**, although there was no significant further reduction of contractions from atropine at higher frequencies (10 & 20Hz, **Table 4.3**).

**Table 4.3: Nerve-mediated contractile responses of control (37°C pre-treatment) porcine denuded detrusor muscle to electrical field stimulation, Data represented as mean ± SEM (n≥10), analysed by a one-way ANOVA with Tukey post-test(\*p<0.05, \*\*p<0.01 vs Initial response)**

Mean ± SEM	1Hz	5Hz	10Hz	20Hz
<b>Initial (mN/g)</b>	28.6 ± 9.0	78.3 ± 20.2	140.9 ± 34.0	228.0 ± 72.0
<b>+ Atropine (1µM)</b> (mN/g)	18.4 ± 6.7	31.3 ± 11.4	37.4 ± 12**	33.5 ± 10.6**
<b>+ Atropine (1µM)</b> <b>+ α,β-mATP</b> <b>(10µM) (mN/g)</b>	3.1 ± 1.6*	12.6 ± 4.8**	15.6 ± 5.7**	17.6 ± 6**

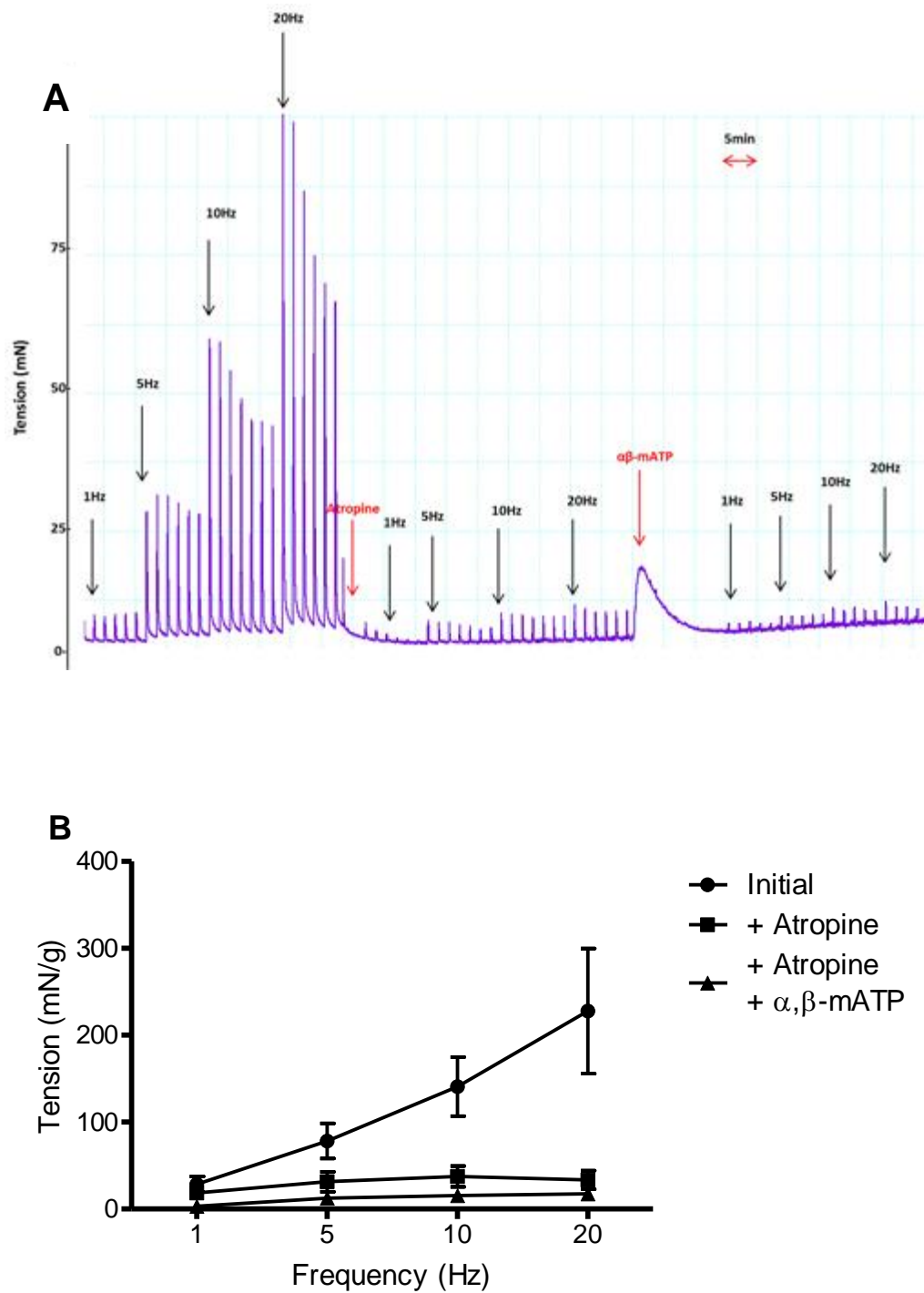


Figure 4.16: A – Representative trace of contractile responses of a porcine denuded detrusor strip to electrical field stimulation (20V, 1ms pulse-width, 5s train). B – Contraction to EFS in the presence of atropine and  $\alpha\beta$ -mATP. Data is represented as mean  $\pm$  SEM ( $n \geq 10$ ), normalised by tissue weight.

At 37°C luminal pre-treatment with gemcitabine (40mg/mL) reduced the contractile response to EFS at all frequencies by 37-45%. Similarly, control porcine tissues pre-incubated at 42°C also displayed reduced nerve-mediated contractions, and synergic effects of gemcitabine and hyperthermia were observed resulting in significantly reduced contractions to EFS across all frequencies with comparison to the control 37°C responses (**Figure 4.17A**).

The addition of atropine (1μM) reduced the EFS contraction by 85% of the initial response at the 20Hz frequency in all tissues, showing acetylcholine having the largest contribution to the EFS mediated contraction in all groups. The effect of combined gemcitabine and hyperthermia pre-treatment significantly reduced the contraction to EFS from control responses at frequencies 10 and 20Hz with atropine (**Figure 4.17B**).

There was no difference between EFS responses with the addition of  $\alpha,\beta$ -mATP between control and tissues pre-treated with gemcitabine and/or hyperthermia (**Figure 4.17C**).

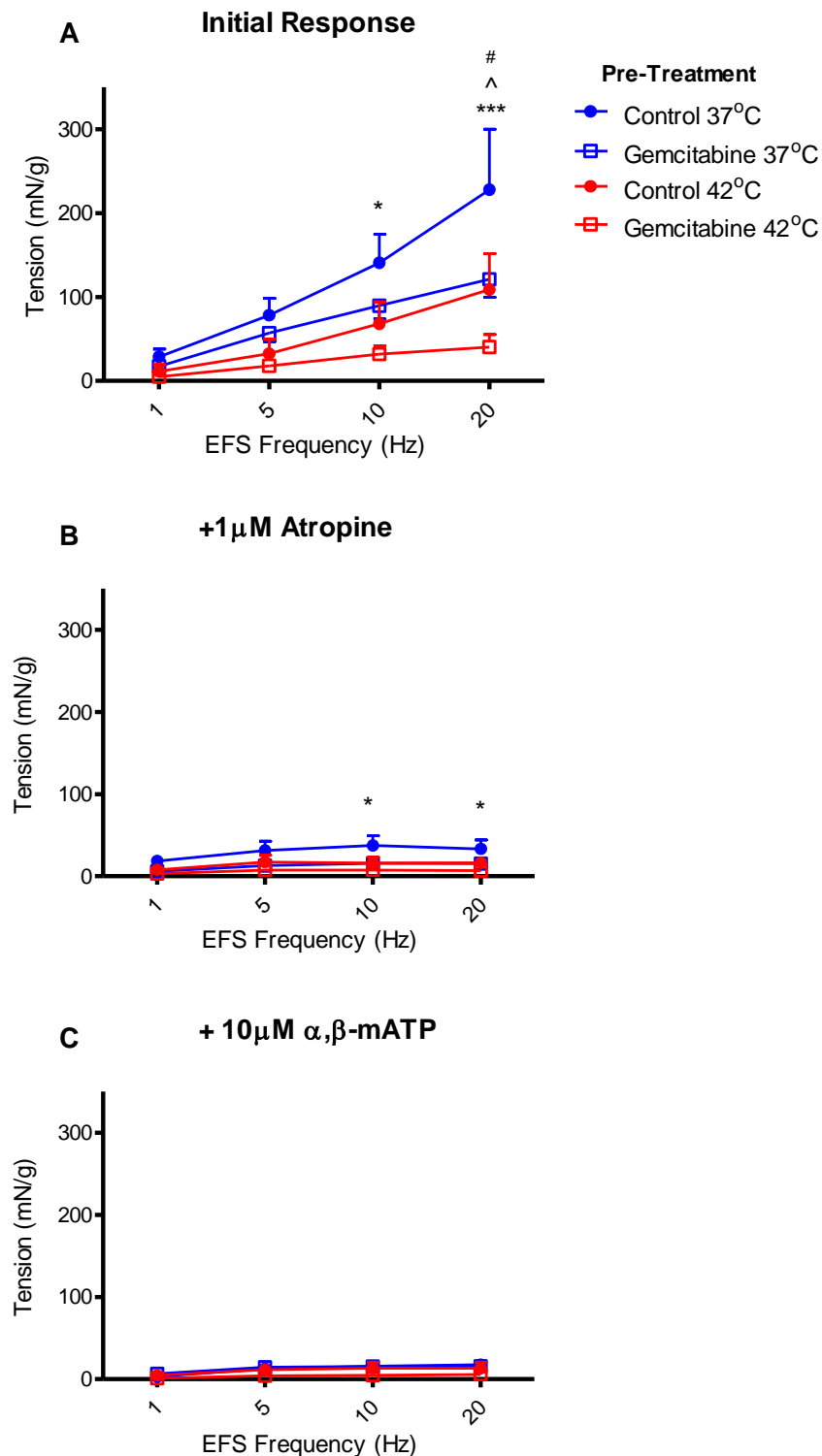


Figure 4.17: Effect of luminal control (0.9% saline) and gemcitabine (40mg/mL) pre-treatment at 37°C and 42°C on contraction of porcine denuded detrusor strips to electrical field stimulation (20V, 1ms pulse-width, 5s train). Data is represented as mean  $\pm$  SEM ( $n \geq 7$ ), analysed by a two-way ANOVA with Bonferroni post-test (# $p < 0.05$  Gemcitabine 37°C vs Control 37°C, ^ $p < 0.05$  Control 42°C vs Control 37°C, \* $p < 0.05$ , \*\*\* $p < 0.001$  Gemcitabine 42°C vs Control 37°C).



## 4.5 DISCUSSION

This study aimed to investigate the effect of pre-incubation with luminal gemcitabine, increased temperature and the combination on the porcine bladder. Intravesical chemotherapy for bladder cancer involves instillation of cytotoxic drugs to lie in direct contact with the urothelium. To mimic this treatment, a modified Ussing's chamber was used to ensure gemcitabine was applied only on the luminal side of the bladder, while the adventitial side was bathed in Krebs-bicarbonate solution. Thus, our model simulated the clinical setting, where tissues are exposed to luminal gemcitabine at a concentration of 40mg/mL and incubated for 60 minutes at either body temperature (37°C) or under hyperthermic conditions (42°C).

### UROTHELIAL EFFECTS OF LUMINAL GEMCITABINE AND/OR HYPERTHERMIA

As the urothelium lies in direct contact with gemcitabine during intravesical treatment, it is likely that damage to the urothelium may in part be responsible for the urological side effects reported by patients. This study examined histological specimens of porcine bladder stained with haematoxylin and eosin after 1hr treatment of gemcitabine incubated at 37°C or 42°C. A decrease in urothelial thickness was observed in porcine tissue treated with gemcitabine, further enhanced by combined hyperthermia and gemcitabine. The distinct crypt-like folded appearance of the urothelium in the control tissues was similar in appearance to that reported in other mammalian urothelium (Desantis et al., 2013). Furthermore, as observed in normal human bladder specimens, a clearly defined basal lamina separates the urothelium from the lamina propria, characterised by smooth contours (Jost et al., 1989). Loss of the tightly folded structure of the urothelium was noted in bladder portions treated with gemcitabine, however the basal lamina remained intact and appeared undamaged by luminal gemcitabine treatment or incubation at either temperature.

Staining with cytokeratin 7, a protein expressed by simple epithelial cells including the urothelium (Moll et al., 1982), showed disruption of the apical membrane in samples of porcine tissue treated with gemcitabine, which was amplified in tissues treated with gemcitabine at 42°C. As previously discussed in Chapter 1, the apical membrane is made up of an arrangement of uroplakin proteins (UP Ia, UP Ib, UP II and UP III) with intervening plaques (Lee, 2011, Apodaca,

2004). Additionally, the presence of a glycosaminoglycan layer (GAG) layer on the apical side of the urothelium is the principle defence barrier against bacteria, proteins and molecules typically present in urine. The GAG layer is made up of chains of disaccharides which attract water molecules and form a gel-like barrier to the urothelium. Recent studies have found that the disaccharide chondroitin sulphate present in the GAG layer plays an important role in the urothelial barrier function (Janssen et al., 2013, Hauser et al., 2009). Furthermore, the combination of chondroitin sulphate and gemcitabine has a synergistic cytotoxic effect on the bladder cell line HT-1376, inducing apoptosis through the activation of capsase 3 and 9 (Ferro et al., 2012). Damage to the GAG layer, in particular the chondroitin sulphate disaccharide, has been linked to the development of interstitial cystitis (characterised by urinary frequency, bladder pain and urgency) and urinary tract infections (Iavazzo et al., 2007, Morales et al., 1996, Hurst et al., 1987, Theoharides et al., 2001, Parsons, 1986). Histological staining of rat urothelium following cyclophosphamide-induced cystitis produced similar disruption to the apical surface of the urothelium, comparable to that observed within this study (Birder and Andersson, 2013). It may be inferred from the results of this study that gemcitabine damages the protective barrier of the urothelium, by inducing apoptosis by chondroitin sulphate expressing cells.

The presence of cytokeratin 20 was also examined on specimens of isolated porcine tissue treated with gemcitabine and the vehicle control. The expression of cytokeratin 20 is restricted to superficial umbrella cells and highly differentiated intermediate cells of the urothelium in normal human bladders (Harnden et al., 1996). It is thought to facilitate membrane transport during bladder filling and contraction (through fusing/internalisation of vesicles to the cytoplasm), and is used as a marker for urothelial dysplasia (Veranic and Jezernik, 2002, Mallofre et al., 2003). The control tissues incubated at 37°C in this study similarly displayed cytokeratin 20 staining restricted to the apical cells of the urothelium. In tissues treated with gemcitabine, there was a distinct decrease in number of cells stained for cytokeratin 20, further exacerbated by combined gemcitabine and hyperthermia pre-incubation. This effect was similarly observed in control tissues incubated at 42°C, indicative of urothelial sloughing by hyperthermia.

While this study provides conclusive evidence of erosion by gemcitabine and hyperthermia pre-treatment of the apical layer of umbrella cells, there was no significant change in urothelial width

measured with histological staining. Witjes et al (2003) noted mild signs of the inflammatory response, and epithelial cellular and nuclear enlargement in pig bladders after repeated intravesical gemcitabine. Additionally, a study investigating the effect of intravesical saline irrigated through the bladder at a temperature of 45°C (temperature of the saline within the bladder was unclear due to crude methodology) noted the cystoscopic appearance of the urothelium as oedematous compared to pre-treatment (Hall et al., 1974). This finding may explain why urothelial sloughing observed after gemcitabine and hyperthermia pre-treatment was not associated with change in the width of the urothelium.

Some conditions of the bladder, including painful bladder syndrome/interstitial cystitis, senescence and spinal cord injury are also associated with changes in the urothelial barrier function (Apodaca et al., 2003, Lavelle et al., 2000, Parsons et al., 1991). Studies profiling urothelial keratin composition in human patients with painful bladder syndrome/interstitial cystitis have found altered cytokeratin 20 expression, with 17% of patients exhibiting an entirely negative stain (Laguna et al., 2006). This study conclusively shows damage to the urothelium by gemcitabine treatment, in particular, sloughing of the apical umbrella cells. As in other pathological bladder conditions, the pain and increased frequency/urgency of urination reported by patients treated with intravesical gemcitabine may be in part due to the compromised barrier function of the urothelium, and the exposure of the underlying bladder nerves and detrusor muscle to irritants and mediators released by the urothelium.

## **UROTHELIAL MEDIATOR RELEASE AFTER GEMCITABINE AND/OR HYPERTHERMIA**

### **PRE-TREATMENT**

Next, the effect of luminal gemcitabine and pre-incubation of porcine tissue at 37°C and 42°C on the function of the urothelium/lamina propria was investigated by measuring the basal and stretch –induced release of the mediators ATP, acetylcholine and prostaglandin E<sub>2</sub>. Release of mediators within this study was measured over a time period of 15 minutes.

In control tissues, there was no change between the basal and stretch-induced release of mediators ATP, acetylcholine or prostaglandin E<sub>2</sub>. Previous studies found that stretch

significantly enhanced the release of ATP from strips of porcine urothelium/lamina propria (Sadananda et al., 2012, Smith et al., 2014, Kang et al., 2013b), but there have been no reports of change in release of acetylcholine and prostanglandin E<sub>2</sub> between basal and stretched states. While this study saw modest increases in ATP release with stretch across all time points measured, this change was not statistically significant. This may be due to slight differences in protocol, namely the size of urothelial tissue tested, the magnitude of stretch stimulus and the time points over which samples were collected.

Pre-treatment with luminal gemcitabine significantly decreased both basal and stretch-induced release of ATP. This is in direct contrast to a previous study by Kang et al (2013b) investigating the effect of the chemotherapeutic doxorubicin on urothelial mediator release. ATP release was significantly enhanced after luminal doxorubicin treatment. While the present study found that gemcitabine sloughed the apical umbrella cells from the urothelium, there was no histologically measured change in urothelial thickness of porcine bladders treated with doxorubicin (Kang et al., 2013b). Doxorubicin has a molecular weight of 589D and it has been estimated that only 3% of the intravesically administered doxorubicin penetrate the GAG-layer. Gemcitabine has a much smaller molecular weight of 299D, which may enable it to penetrate the apical membrane of the bladder urothelium. In addition, the urothelial erosion and hence fewer urothelial cells, observed after gemcitabine treatment may account for the depressed ATP release with comparison to control.

Ferguson and colleagues (1997) first reported the release of ATP from the serosal, but not mucosal surface of the bladder. Further investigation has since examined the relative contribution of this release from isolated urothelium from the mucosal (umbrella cell) and serosal (basal cell) surfaces using Ussing's chambers (Wang et al., 2005). ATP release with hydrostatic pressure was enhanced with stretch from both sides of the urothelium, the amount released into the apical hemichamber was approximately 50-fold greater than that released into the serosal hemichamber. The gemcitabine-induced stripping of the umbrella cells of the urothelium may be responsible for the depressed release of ATP seen in the present study. As damage to the umbrella cell layer induces functional and structural changes to the underlying cells to restore the barrier properties of the urothelium (Kreft et al., 2009a, Kreft et al., 2009b,

Lavelle et al., 2002), it would be interesting to investigate the release of ATP from gemcitabine-treated urothelium/lamina propria after cellular recovery.

Interestingly, control porcine tissues pre-incubated at 42°C released less ATP measured basally and during stretch. Histology and staining for cytokeratin 7 and 20 of this tissue also displayed sloughing of the apical layer of umbrella cells, which may account for the depressed release of ATP measured in this study.

As previously described, there was no enhanced release of acetylcholine from the urothelium/lamina propria with stretch. This concurs with the results of previous studies quantifying urothelial mediator release (Kang et al., 2013b, Smith et al., 2014). Both basal and stretched release of acetylcholine accumulated in a time dependent manner in control cells incubated at both 37°C and 42°C. Pre-treatment with gemcitabine at incubation temperature of 37°C did not change the manner of release, increasing significantly from initial basal release after 15 minutes. However, there was no difference between acetylcholine release of control and gemcitabine treated tissues, nor did hyperthermia pre-treatment have any effect.

Finally, prostaglandin E<sub>2</sub> release was measured from strips of isolated urothelium/lamina propria. While no difference of prostaglandin E<sub>2</sub> release was observed between basal or stretched conditions in control tissues, a time-dependent accumulation was evident. Gemcitabine pre-treatment significantly enhanced release of prostaglandin E<sub>2</sub>, although it was similarly accumulated in a time-dependent manner.

Prostaglandins are formed from arachidonate by the action of cyclooxygenase (COX) enzymes, and prostaglandin E<sub>2</sub> in particular has been found to be released from both the detrusor and urothelium, contributing to the basal tone of the detrusor muscle and activating afferent nerves. It is well documented that enhanced release of prostaglandins play a role in mediating the inflammatory response within tissues (Ricciotti and FitzGerald, 2011). Patients with overactive bladder symptoms have significantly increased urinary levels of prostaglandin E<sub>2</sub> (Kim et al., 2005, Kim et al., 2006). Using a rat model of acute (4 hours) and chronic (10 days) cyclophosphamide-induced cystitis, Hu et al. (2003b) found that COX-2 mRNA was increased 12- and 9-fold respectively in acute and chronically inflamed bladders. COX-2 protein

expression paralleled the mRNA profile, and prostaglandin E<sub>2</sub> release was also significantly increased from the rat bladder. Interstitial cystitis is characterised by increased frequency and painful urination, similar to the symptoms reported by patients undergoing intravesical gemcitabine treatment.

A further investigation of the role of prostaglandins on sensory nerve activity found that the addition of loxoprofen (a cyclooxygenase inhibitor) and ONO-8711 (prostaglandin E<sub>2</sub> receptor 1 antagonist) inhibited the enhanced neuronal activity in inflamed rat bladders (Ikeda et al., 2006). As prostaglandin E<sub>2</sub> was enhanced with gemcitabine pre-treatment in porcine tissues, the above evidence supports its role in inducing an inflammatory response within human patients undergoing intravesical therapy with gemcitabine. This may explain the symptoms of overactivity and painful urination in this treatment.

Basal release of prostaglandin E<sub>2</sub> from control tissues pre-incubated at hyperthermic conditions (42°C) was enhanced from the 37°C tissue response. Pyrexia induced prostaglandin E<sub>2</sub> is dependent on COX-2 enzyme activity in both mice and humans (Schwartz et al., 1999, Li et al., 1999). This indicates that the increased formation of prostaglandin E<sub>2</sub> in hyperthermic conditions may result from increased COX-2 activity. Due to this enhanced release in hyperthermic control tissues, the enhanced release of prostaglandin E<sub>2</sub> with gemcitabine and hyperthermia pre-treatment was not observed.

There was no change in stretched conditions in control tissues between the 37°C and 42°C incubation temperatures. However, release of prostaglandin E<sub>2</sub> from gemcitabine pre-treated tissues at 42°C was enhanced from the 37°C gemcitabine group, possibly a synergistic effect from both heat and gemcitabine inducing enhanced release of prostaglandin E<sub>2</sub>.

## **EFFECT OF GEMCITABINE AND/OR HYPERTHERMIA ON CONTRACTILE AND RELAXANT RESPONSES OF THE BLADDER**

This study shows conclusive evidence that luminally applied gemcitabine and the effect of increased temperature causes damage to the urothelium, and alters the release of urothelial mediators ATP and prostaglandin E<sub>2</sub>. However, the micturition cycle of bladder filling and emptying is dependent upon the contraction and relaxation of the detrusor muscle. Furthermore, bladder specimens from patients with painful bladder syndrome and idiopathic detrusor overactivity are correlated with overexpression of M<sub>2</sub> and M<sub>3</sub> muscarinic receptors and a phenotype of increased frequency and urgency respectively (Mukerji et al., 2006). Anti-muscarinics are considered the first-line treatment for patients with overactive bladder, inhibiting muscarinic receptor-induced contractions of the bladder, and recently suggested to block bladder afferent nerve activity (Abrams et al., 2006, Yamaguchi, 2010). A further treatment for overactive bladder is  $\beta$ -adrenoceptor agonists, which act to improve the storage capacity of the bladder by inducing relaxation of the detrusor muscle, suppress involuntary detrusor contraction and improve symptoms of increased frequency (Yamaguchi and Chapple, 2007). Furthermore, it has recently been demonstrated that  $\beta$ -AR activation by isoprenaline in rat urothelial cells can trigger production and release of nitric oxide due to increased Ca<sup>2+</sup> following stimulation of the adenylyl cyclase pathway in urothelial cells (Birder et al., 2002). This NO release is suggested to further relax the detrusor muscle and alter the perceptions of bladder sensation by acting on the bladder afferent nerves (Pandita et al., 2000).

The following sections will elucidate the effects of luminal pre-treatment with gemcitabine and/or hyperthermia on the contractile and relaxant responses of the denuded detrusor, intact tissue and isolated urothelium.

### **DETRUSOR MUSCLE**

During voiding, contraction of the detrusor muscle is initiated by the efferent nerves of the bladder. As previously discussed, parasympathetic postganglionic fibres release acetylcholine, acting upon the M<sub>3</sub> muscarinic receptors in the detrusor muscle to cause contraction. In addition, parasympathetic stimulation causes the release of non-adrenergic, non-cholinergic (NANC)

transmitters which similarly induce contraction of the bladder smooth muscle. ATP was first implicated as a NANC transmitter through early experiments of the gut and later confirmed within the bladder (Burnstock et al., 1970, Kasakov and Burnstock, 1982).

This study investigated the contribution of muscarinic and purinergic receptors to nerve mediated contractions induced by EFS. In the presence of the neurotoxin TTX, no contraction of the bladder tissue was recorded during preliminary experiments, confirming responses to these EFS parameters were nerve induced. The addition of atropine (a muscarinic antagonist) significantly depressed contraction to EFS at frequencies above 10Hz, reducing contraction at 20Hz by 85%. This was similarly found in the porcine bladder by Sibley (1984), who reported atropine was least effective at blocking nerve-mediated contraction at low frequencies, while only 17% of contraction persisted at high frequency stimulation. Contraction of the healthy human detrusor muscle is considered to be almost purely cholinergic, however atropine-resistance in nerve mediated contractions from human detrusor have been reported in patients with interstitial cystitis, unstable and obstructed bladders (Palea et al., 1993, Sibley, 1984, Bayliss et al., 1999). Contraction of these tissues is abolished in the presence of  $\alpha,\beta$ -mATP, indicative of a role of enhanced neurally released ATP in contraction of the pathological bladder (Bayliss et al., 1999).

ATP is co-released with acetylcholine from the bladder nerves, acting upon the P2X (ion channel family) and P2Y (G-protein coupled receptor family) purinoceptors present in the detrusor muscle. The purinergic agonist  $\alpha,\beta$ -mATP acts to stimulate and then desensitize the P2X-purinoceptors. The addition of this agonist with atropine to the strips of denuded detrusor almost abolished the contraction to EFS at low frequencies, but had only a small further inhibitory effect on high frequency-induced contractions.

This indicates that there may be a role of  $\alpha,\beta$ -mATP resistant purinergic receptors within the porcine detrusor muscle that mediate contraction to neural stimulation. The presence of P2Y receptors within the porcine bladder detrusor have recently been confirmed, and it has been demonstrated that direct stimulation of these receptors in mouse bladder induced increases in bladder smooth muscle tone (Bahadory et al., 2013, Yu et al., 2013). Therefore, the atropine,



$\alpha,\beta$ -mATP-resistant contractions of the porcine bladder measured within this study may be mediated by ATP acting upon P2Y purinergic receptors.

Pre-treatment with gemcitabine and/or hyperthermia depressed contraction to EFS at all frequencies tested. A significant reduction in contraction amplitude was measured in the combined gemcitabine and hyperthermia pre-treated group above 5Hz. This result is suggestive of the role of gemcitabine in reducing the nerve-induced contractions of the bladder; however the contraction of the detrusor muscle to endogenous agonists was also reduced, as discussed below.

The contraction of the denuded detrusor muscle to KCl was reduced with gemcitabine pre-treatment. This was significant in the combined gemcitabine and hyperthermia group, where contraction was reduced by approximately 80%. Contraction to cumulative additions of carbachol and ATP were similarly reduced by gemcitabine and/or hyperthermia pre-treatment in the denuded detrusor tissue. Furthermore, the maximal response to isoprenaline was reduced by 50-85% with gemcitabine and/or hyperthermia pre-treatment. However, there were no differences in the potency of responses of both carbachol and isoprenaline of the denuded detrusor. This is indicative of the loss of contractile and relaxation capacity of the detrusor muscle with gemcitabine and/or hyperthermia treatment. Similarly, the cholinergic and P2X-mediated components of contraction stimulated by EFS were unchanged by gemcitabine and/or hyperthermia treatment. Therefore, it seems that gemcitabine reduces the contractile activity of the detrusor muscle, rather than altering the activity of efferent bladder nerves. However, whether this is a transient or prolonged effect was not measurable in the present study, as tissue viability was compromised after ~16 hours.

The extent to which gemcitabine can penetrate the urothelium and reach the underlying lamina propria and detrusor muscle is currently unknown. Luminal pre-treatment with the chemotherapeutic doxorubicin enhances efferent nerve activity, without directly affecting the detrusor muscle which is in contrast to findings of the present study (Kang et al., 2013b). However, only ~3% of intravesically administered doxorubicin permeates through the urothelium (Wientjes et al., 1996), while the penetrative effect of gemcitabine through the bladder wall is unknown. One of the mechanisms of increased cytotoxicity associated with combined

hyperthermia and intravesical chemotherapy for bladder cancer is increased cellular membrane permeability (Lammers et al., 2011), and this study has shown that gemcitabine strips the apical layer of umbrella cells. Gemcitabine may directly induce cellular damage on the detrusor muscle, or allow the permeation of noxious substances from the urine through the urothelium, thus resulting in underactive or impaired contractile function.

## INTACT TISSUE

The presence of an intact urothelium/lamina propria on isolated porcine bladder strips has been found to inhibit contractions induced by the muscarinic agonist carbachol and KCl (Hawthorn et al., 2000, Wuest et al., 2005). In human bladder tissue, both KCl and carbachol-induced and nerve-mediated contractions are significantly inhibited by the presence of the urothelium, but not neurokinin A (part of the tachykinin family of neurotransmitters) (Chaiyaprasithi et al., 2003, Propping et al., 2013).

In the present study, the contractile response of the intact control tissue (37°C) to KCl and ATP were significantly lower than the denuded detrusor contractile magnitude. As discussed in Chapter 1, the urothelium of intact porcine bladder tissue strips releases a diffusible inhibitory factor (UDIF) in response to muscarinic stimulation and the addition of KCl, which inhibits the contraction of the underlying detrusor (Hawthorn et al., 2000, Wuest et al., 2005). Similarly, removal of the urothelium from rat isolated bladder rings increased the contractile response to ATP (Khattab and Al-Hrasen, 2006). In the present study, the presence of the urothelium/lamina propria reduced the responses of the detrusor by 50-65% in control tissues (37°C) to carbachol, KCl and ATP, correlating with the inhibitory effect of the urothelium reported within these studies.

Furthermore, Masungaga et al (2010) investigated whether the presence of the urothelium had any effect on relaxation responses to  $\beta$ -adrenoceptor agonists of the detrusor muscle, finding no differences between maximal responses of intact and denuded detrusor, similar to the results of the present study. However, it was found by Murakami et al (2007) that isoprenaline was more potent at inhibiting porcine detrusor contractions induced by carbachol in the presence of the urothelium. From this observation, it was suggested that the urothelium releases UDIF in response to isoprenaline stimulation, as it was confirmed not to be mediated by nitric oxide (Murakami et al., 2007). The mechanism of action of UDIF has not yet been identified, however

its effects are not prevented with inhibition of nitric oxide synthase, cyclo-oxygenase production, catecholamines, adenosine, GABA, purinergic receptors, potassium channels or endothelium-derived hyperpolarizing factor are inhibited (Chaiyaprasithi et al., 2003, Hawthorn et al., 2000, Masunaga et al., 2010). In the present study, the inhibitory effect of the urothelium to cholinergic contraction of the detrusor was observed, although similar to previously reported studies, there was no enhanced relaxing effect. This supports the involvement of UDIF, which inhibits contraction but does not change relaxation of the detrusor muscle.

Interestingly, the inhibitory effect of the urothelium with contraction to KCl, ATP and carbachol was not observed in gemcitabine pre-treated tissues at either temperature. Additionally, in comparison to controls, gemcitabine and/or hyperthermia pre-treatment generally attenuated intact tissue responses to KCl, ATP, carbachol and isoprenaline. However this effect was only significant in the maximal contraction to carbachol, although there was no change in the potency of this response.

Taken together, it would appear that luminal gemcitabine has a significant effect on the release of the urothelial-derived inhibitory factor. This may be a result of altered urothelial function due to physical damage to the urothelium of porcine bladders that was shown in the present study with luminal gemcitabine. A previous study by Smith et al (2014) observed that the inhibitory effect of the urothelium on porcine detrusor contraction was significantly reduced following luminal DMSO. Furthermore, reduction in inhibitory mechanisms of the urothelium on contraction of the detrusor muscle have similarly been observed in the human neurogenic overactive bladder (Chess-Williams et al., 2009). Consequently, the observed reduction in urothelial inhibition of the detrusor after gemcitabine pre-treatment as seen in the present study may contribute to the symptoms of overactive bladder as reported by patients undergoing intravesical therapy with this agent.

#### ISOLATED UROTHELIUM/LAMINA PROPRIA

This study shows conclusive evidence that luminally applied gemcitabine and the effect of increased temperature causes damage to the urothelium, and alters the release of urothelial

mediators ATP and prostaglandin E<sub>2</sub>. The urothelium and underlying lamina propria have been confirmed in recent years to play a significant role in normal bladder functioning, releasing factors that modulate detrusor contraction and sensory nerve activity (Hawthorn et al., 2000, Birder, 2011). It has previously been shown that the urothelium has contractile properties distinct from the detrusor muscle, suggested to be mediated by the suburothelial myofibroblasts (Sadananda et al. 2008). However, it has also been suggested by Heppner et al. (2011) that contraction of the urothelium/suburothelium may be generated by a component of muscularis mucosae in the suburothelium that is distinct from the detrusor.

Furthermore, the urothelium/lamina propria has been found to exhibit spontaneous phasic activity and contracts to carbachol, mediated by the M3 muscarinic receptor (Moro et al., 2011). There is some evidence that indicates that this spontaneous activity of the urothelium may drive detrusor muscle activity. In the rat bladder, calcium and membrane potential transients produced by carbachol begin at the urothelial interface before spreading to the detrusor (Zygmunt et al., 1993). This effect is enhanced in overactive bladders from cats with feline cystitis, also associated with super sensitivity to muscarinic stimulation in the urothelium/lamina propria (Ikeda et al., 2009)

As the urothelium is damaged by gemcitabine and the effect of hyperthermia, it is logical to assume that the contractile component of the urothelium will also be altered. The spontaneous phasic contractions were examined in isolated urothelium/lamina propria and denuded detrusor after pre-treatment with gemcitabine and/or hyperthermia. Both the amplitude and frequency of contractions were significantly less in the denuded detrusor strips compared to the isolated urothelium/lamina propria across all tissue groups. Moro et al. (2011) first reported a significantly lower rate of spontaneous contractions in the detrusor muscle compared to the urothelium/lamina propria, similar to the findings of the present study. Gemcitabine pre-treatment had no effect on the spontaneous contraction of the tissues, nor did pre-incubation with saline at 42°C, although a trend of increasing frequency on contractions was observed with gemcitabine treatment. In spinal cord transected mice with bladder overactivity, Ikeda and Kanai (2008) found bladders exhibited significantly enhanced amplitude and frequency of spontaneous contractions, suggested to be associated with the over-expression of muscarinic

receptors in the urothelium/lamina propria. This effect was measured two weeks after spinal cord transection, while in the present study, spontaneous activity was measured immediately after gemcitabine pre-treatment. The development of enhanced spontaneous contraction of the urothelium after intravesical gemcitabine in patients over a prolonged period may contribute to the symptoms of increased frequency of urination.

This study also investigated the contraction of the isolated urothelium/lamina propria to KCl, ATP and carbachol, and the relaxant response mediated by isoprenaline. While gemcitabine and/or hyperthermia pre-treatment did not change the response of the urothelium/lamina propria to KCl and ATP, contraction to carbachol and relaxation to isoprenaline were attenuated. This result was not unexpected, as the urothelial sloughing that was observed after luminal gemcitabine pre-treatment most likely altered the contractile and relaxation component of the urothelium as well.

## **CONCLUSIONS**

A previous study by Kang et al. (2013b) investigated the effects of luminal doxorubicin on porcine bladder using similar protocols to the present study. Similar to treatment with gemcitabine, the side effects of intravesical doxorubicin for NMIBC reported by patients include symptoms of painful and overactive bladder (Koya et al., 2006). Interestingly, pre-treatment with doxorubicin on porcine tissue increased basal urothelial ATP release, enhanced the urothelium/lamina propria contraction to muscarinic stimulation and enhanced neurogenic responses of the detrusor muscle (Kang et al., 2013b). Taken together, these changes in bladder function induced by doxorubicin were suggested to lead to bladder overactivity through a combination of sensitization of afferent nerves, and the contractile component of the detrusor muscle through enhanced neurogenic responses.

This is in contrast to the results of the present study, where pre-treatment of gemcitabine on porcine tissue generally depressed the neurogenic and myogenic responses, although enhanced urothelial prostaglandin E<sub>2</sub> release and abolished the effect of UDIF. From this, it appears that the symptoms of bladder overactivity are similarly induced by sensitized afferent

nerves due to enhanced urothelial mediator release, and dysfunction of the inhibitory effect of the urothelium on the detrusor muscle, likely due to direct urothelial sloughing. The depressed contractile response of the detrusor muscle to endogenous muscarinic and purinergic stimulation seen in the present study mimic the actions of the current treatments for overactive bladder. However, the reduced response to efferent nerve stimulation after gemcitabine treatment may also contribute to the symptoms of increased frequency and urgency, as this has similarly been observed in the overactive detrusor muscle from obstructed and idiopathic unstable bladders (Sibley, 1997, Steers, 2002).



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## **CHAPTER 5:**

### **EFFECTS OF GEMCITABINE IN VIVO AND ON ISOLATED WHOLE BLADDER FUNCTION IN MICE**

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## 5.1 INTRODUCTION

The previous chapter investigated the immediate effects of intravesical gemcitabine on the porcine urothelium. However, chemotherapy for bladder cancer in human patients is given as a repeated schedule, with periods of recovery between treatments. This chapter investigates the effect of single and repeated doses of intravesical gemcitabine and subsequent recovery periods in mice.

### TREATMENT SCHEDULE

As described previously, doses of 2000mg gemcitabine administered in 50mL of 0.9% saline (40mg/mL) has proven efficacy against superficial bladder cancer and is well tolerated in humans (Addeo et al., 2010, Mattioli et al., 2005). A study reported improved tumour response rates with intravesical gemcitabine given as repeated instillations, with complete remission rates reported in 44% of patients undergoing 6 weekly doses, compared to 40% with 3 bi-weekly doses and 10% undergoing a single post-operative transurethral resection dose (Gardmark et al., 2005). Furthermore, multiple Phase I, II and III studies have examined the tolerability of treatment schedules using intravesical gemcitabine in patients with non-muscle invasive bladder cancer (NMIBC) (Shelley et al., 2012). **Table 5.1** highlights the incidence following urological side effects of intravesical gemcitabine at a dosage of 40mg/mL given once weekly for 6 weeks.

As the weekly treatment schedule of 2000mg/50mL (40mg/mL) gemcitabine has been proven to be the most effective regime in human patients with NMIBC, the present study investigated the effect of intravesical treatments in mice. Ethical approval was obtained from the Griffith University Ethics Committee for single and repeated intravesical (2 instillations) treatments on a once-weekly schedule in mice.

**Table 5.1: Local toxicity reports of patients undergoing treatment schedules with intravesical gemcitabine**

Study	Participants	Gemcitabine Schedule	Toxicity & Side Effects
<b>Gontero et al. (2004) Phase II study</b>	39 patients with Ta or T1 (grade 1 & 2) NMIBC patients	2000mg/50mL saline, once/week for 6 weeks	15% Hematuria 38% Dysuria 8% Frequency 8% Urgency
<b>De Berardinis et al. (2004) Phase I study</b>	12 patients with Ta or T1 NMIBC patients	500, 1000, 1500 or 2000mg/50mL saline (3 patients/group), once/week for 6 weeks	1 (out of 3) patient treated with 2000mg reported frequency, urgency and dysuria
<b>Bartoletti et al. (2005) Phase II study</b>	116 patients with intermediate to high risk NMIBC	2000mg/50mL saline, once/week for 6 weeks	22 patients (19%) reported frequency, urgency, dizziness and abdominal pain
<b>Addeo et al. (2010) Phase III study</b>	54 patients with Ta or T1 (grade 1-3) NMIBC	2000mg/50mL saline, once/week for 6 weeks	38.8% patients reported side effects including dysuria, suprapubic pain, haematuria and cystitis

## COMPARISON OF HUMAN AND MOUSE BLADDER FUNCTION

The structural anatomy of bladders in mice and humans are comparable. Similar to the human urinary system, the urothelium lines the luminal side of the bladder, sitting atop the suburothelium/lamina propria and detrusor muscle layers. Although the urothelium in murine bladders are only 2-3 cell layers thick (compared to 5-7 in humans), these layers are morphologically distinct and similarly play a role in sensory transmission (Treuting et al., 2012). Furthermore, the superficial layer of the urothelium expresses both uroplakins and glycosaminoglycans (GAG) in similar patterns to human tissue (Ramesh et al., 2004, Aboushwareb et al., 2009).

There are similarities and differences in the physiological functioning of the mouse bladder with comparison to human tissue. As in human tissue, the detrusor of mouse bladders similarly

display spontaneous contractile activity, and the presence of the urothelium similarly attenuates the amplitude of detrusor contraction (Meng et al., 2008, Sibley, 1984).

Contraction to muscarinic stimulation is also mediated via the  $M_3$  receptor in the mouse, confirmed through the lack of cholinergic contraction to carbachol in  $M_2/M_3$  knock-out mice (Matsui et al., 2002). However, the contribution of  $Ca^{2+}$  influx in detrusor contraction differs between the human and mouse species (Choppin and Eglen, 2001, Wuest et al., 2007). The relaxation of the bladder during filling is mediated via the release of noradrenaline from sympathetic nerves acting on the  $\beta$ -adrenoceptors present in the detrusor muscle. In humans, the relaxation to the  $\beta$ -agonist isoprenaline is mediated via the  $\beta_3$  subtype, while in mice, antagonism of the  $\beta_2$ -AR abolishes the response to isoprenaline, indicating that this receptor facilitates relaxation (Igawa et al., 2001, Igawa et al., 1998, Wuest et al., 2009).

As discussed in previous chapters, nerve-mediated contraction in human and porcine bladders is almost completely mediated via the release of acetylcholine from the parasympathetic nerves (Sibley, 1984). In comparison, stimulation of the parasympathetic nerves in the mouse bladder causes the co-release of acetylcholine and ATP, inducing contraction through the muscarinic and purinergic receptors respectively, similar to the human bladder in pathological conditions. *In vitro* and *in vivo* studies on rodents have shown that contraction to EFS is mediated significantly by non-cholinergic, non-adrenergic stimulation (Igawa et al., 1993, Hegde et al., 1998). In mice, the purinergic component of contraction to electrical field stimulation has been reported to account for 55% of the nerve-mediated response (Lamarre et al., 2014). Furthermore, the P2X receptor-mediated component accounts for approximately 70% of the EFS response to short trains of stimuli (1s trains), as determined in P2X<sub>1</sub> receptor deficient mice (Vial and Evans, 2000). However, the purinergic component of human bladder contraction is enhanced in diseased states, and this response is mediated by ATP acting on the P2X<sub>1</sub> receptors in both species (Elliott et al., 2013, Vial and Evans, 2000).

## THE EFFECT OF INTRAVESICAL GEMCITABINE ON MOUSE BLADDER FUNCTION

To the best of my knowledge, no studies have investigated the functional effect of intravesical gemcitabine on mouse bladders. A study involving treatment of the mouse bladder tumour (MBT-2) in female C3H/eb mice with 6-weekly instillations of gemcitabine (0.5, 2.5 and 10mg) reported no statistical differences in the histological changes of the bladder wall, including mucosal atypia, mucosal oedema, epithelial hyperplasia, mucosal erosion, sub-mucosal fibrosis and muscle thickness (Nativ et al., 2004).

Bladder damage in mice after single and repeated intravesical instillations has been assessed using the chemotherapies mitomycin C and doxorubicin (Post et al., 1993). Bladder function was assessed as changes in urinary frequency, with both drugs inducing three-fold increases in the frequency of urination during weekly-treatments of 1mg/mL (mitomycin C) and 6mg/mL (doxorubicin). However, the above investigation into the effects of mitomycin C and doxorubicin did not report the mechanisms underlying the increased voiding frequency (Post et al., 1993). It has been shown that drugs specifically toxic (eg. cyclophosphamide) to the bladder induce rapid epithelial loss and an inflammatory response, causing functional bladder changes (Stewart, 1986).

A further study by Post et al. (1995b) noted acute functional damage in 80% of mice treated with intravesical mitomycin C, and that the mitotic activity of bladder epithelial cells was markedly increased 3 weeks after intravesical mitomycin C with comparison to controls, indicative of cell growth. This suggests damage to the epithelial lining of the bladder by intravesical instillation of cytotoxic drugs and the corresponding cell recovery mechanisms may account for the symptoms of increased voiding frequency in mice. Further investigation of the mechanisms of the functional changes of the bladder after intravesical chemotherapy is required to elucidate appropriate treatment or preventative regimes.

Cyclophosphamide (CPO) and ifosfamide (IFO) are chemotherapeutic drugs commonly used for the treatment of a variety of cancers, including breast, testicular, lung, cervical and lymphomas (Nichols, 1995, Buda et al., 2003, Gianni et al., 1990, Loehrer, 1990). It is well reported that systemic treatment with these drugs leads to bladder toxicity, bladder pain and dysfunction manifested as increased frequency and urgency of urination and dysuria (Korkmaz et al., 2007,

Fukuoka et al., 1991), not dissimilar to that reported by patients undergoing intravesical chemotherapy for bladder cancer. Rodents treated with CPO or IFO similarly demonstrate increased urinary frequency and decreased voiding pressure (Boudes et al., 2011, Macedo et al., 2011). Contraction of the bladder to muscarinic stimulation was depressed after systemic CPO/IFO treatment in rats (Macedo et al., 2011, Giglio et al., 2005), restored to control responses with the addition of the nitric oxide synthase inhibitor L-N<sup>G</sup>-Nitroarginine (L-NNA). Furthermore, animal models of bladder overactivity treated with CPO have also shown reduced detrusor contraction to P2X stimulation (Mok et al., 2000). These changes in bladder contractile responses have been suggested to play a role in the symptoms of bladder overactivity induced by CPO and IFO treatment. These parameters will be examined within this study to investigate the changes in bladders of mice induced by intravesical gemcitabine.

## EXPERIMENTAL PROTOCOLS

Bladder cancer is often considered a disease of the elderly, with over 90% of Australian patients diagnosed over the age of 50 (Government, 2015). It is also well established that lower urinary tract symptoms increase in frequency with aging (Diokno et al., 1986, Diokno et al., 1992). In both men and women, post void residual volume increases, while bladder capacity and peak flow rate decrease with age (Madersbacher et al., 1998). It has been suggested that fibrosis of the bladder wall or alterations in nerve activity with aging are responsible for the changes in functional capacity, as the detrusor contraction strength in both men and women is preserved with age (Madersbacher et al., 1998, Malone-Lee and Wahedna, 1993). Symptoms of bladder overactivity, including increased voiding frequency, detrusor hyperexcitability and sensory stimulation have similarly been reported within aged mice (Triguero et al., 2014, Daly et al., 2014). To account for these physiological changes in patients, an aged mouse model (female ex-breeders) was chosen, approximately 32-weeks in age.

The human urinary system exhibits variations in urine output over a 24-hour period, coinciding with the day/night cycle. The circadian variations in urination patterns depend on daily urine production, the physical properties of the bladder and the neural control (Colwell, 2015). Rodents, including mice, are a nocturnal species. Mice demonstrate a circadian rhythm in

micturition frequency and volume, in part driven by the urine production by the kidney, which is linked to the circadian clock (Zuber et al., 2009, White et al., 2014). It has recently been reported that urinary bladder smooth muscle exhibit no diurnal differences in KCl or nerve-evoked contractile properties (White et al., 2014). This is of significance as experiments in the present study were performed during the day.

Voiding pattern analysis using filter paper has been confirmed in studies appearing in the literature as a valid method of analysis of voiding behaviours in rodents (Sugino et al., 2008). While the diurnal rhythm of bladder capacity and voiding frequency/volume is well documented, multiple studies have reported comparable micturition patterns during analysis in the day between mouse species and gender using the filter paper method (Sugino et al., 2008, Uvin et al., 2013, Daly et al., 2014).

In humans, evaluation of bladder function is performed through cystometry, which records bladder filling/emptying and the consequent changes in intravesical pressure. The previous chapter using porcine bladder tissue strips is an excellent model to determine the components of bladder contraction/relaxation; however a more physiologically relevant model is needed to assess the coordination between intact urothelium, underlying suburothelium containing bladder nerves and the detrusor muscle in its original form. Fabiyi and Brading (2006) validated the use of a whole-bladder model, which involved catheterisation of female mouse bladders through the urethra, measuring responses to various agonists and nerve stimulation as changes in intravesical pressure during an isovolumetric condition. Responses were found to be comparable to muscle strips, confirming the use of this model as an effective technique for assessing bladder functioning. Consequently, the isolated whole bladder experimental protocol was used within this study, similarly using female mice to investigate the effects of pre-treatment with intravesical gemcitabine.

## 5.2 Aims

The aims of the present study were to investigate the effects of *in vivo* single and repeated intravesical gemcitabine (40mg/mL) instillations on the mouse urinary bladder. Specific aims were:

1. To investigate the changes in voiding patterns with single and repeated intravesical gemcitabine instillations in mice
2. To determine the effect of single and repeated intravesical gemcitabine instillations in mice bladders on the histological appearance of the urothelium
3. To measure the release of ATP, acetylcholine and prostaglandin E<sub>2</sub> from the urothelium and serosa of mouse bladders treated with single or repeated intravesical gemcitabine instillations
4. To examine the effect of single and repeated intravesical gemcitabine instillations on spontaneous bladder contractions and responses to muscarinic, purinergic and adrenergic receptor stimulation
5. To determine the effects of single and repeated intravesical gemcitabine instillations in mice bladders on the nerve-mediated contractions and assess the contributions of the nitric oxide, cholinergic and adrenergic components



## **5.3 MATERIALS AND METHODS**

### **ETHICAL APPROVAL**

Approval for this project was obtained from the Griffith University Animal Ethics Committee (GU Ref No: MSC/07/14/AEC) held by Dr Roselyn Rose'Meyer. Ethical considerations in keeping numbers of animals sacrificed to a minimum meant a control group (no intravesical treatment) was not approved and not investigated.

### **ANIMALS**

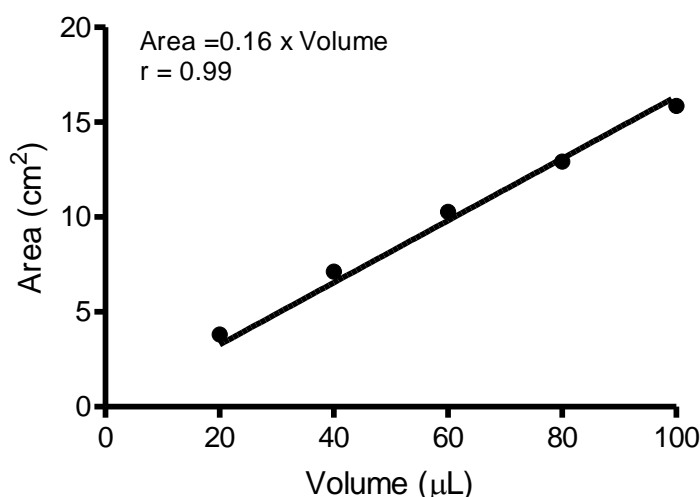
Adult female C57BL/6JArc mice were obtained from the Animal Resource Centre (Canning Vale, WA, Australia) and housed in the Griffith University Animal House. Animal treatments and euthanasia were performed at the Griffith University Animal House and tissues were transported to Bond University for all experimental procedures (Bond University ethics RO1762).

The female mice were ex-breeders, approximately 32-weeks in age. These mice were selected due to ease of intravesical treatment in females, and as a model of aged subjects. The C57BL/6JArc is a widely used inbred strain of mice. The animals were housed for one week prior to handling in a controlled environment with 12 hour light/dark cycles, temperature 23°C with free access to food and water.

### **VOIDING PATTERN ANALYSIS**

The voiding pattern of the mice was examined before and after treatment using Whatman Filter paper #1 (Interpath Services, Victoria, Australia). Mice were housed singly in cages lined with 15 x 21cm sheets of Whatman Filter paper #1 for 4 hours with free access to food and water. The filter paper was collected and urine spots detected using a Molecular Imager ChemiDoc XRS ultraviolet transilluminator (#720BR1293 BioRad, California USA). The papers were photographed, digitised, and analysed using Image J software. This protocol was based upon previously reported studies into the voiding patterns of aged and overactive bladder models in mice (Uvin et al., 2013, Daly et al., 2014, Boudes et al., 2011). Voiding analysis was performed

on the mice before intravesical treatment and was repeated 24hrs after treatment, before euthanasia. All voiding pattern analysis were performed in the morning, beginning at the change-over of the light/dark cycle (7.30am). Voided volume was determined by measuring the area of urine patches of known volume, shown by **Figure 5.1**.



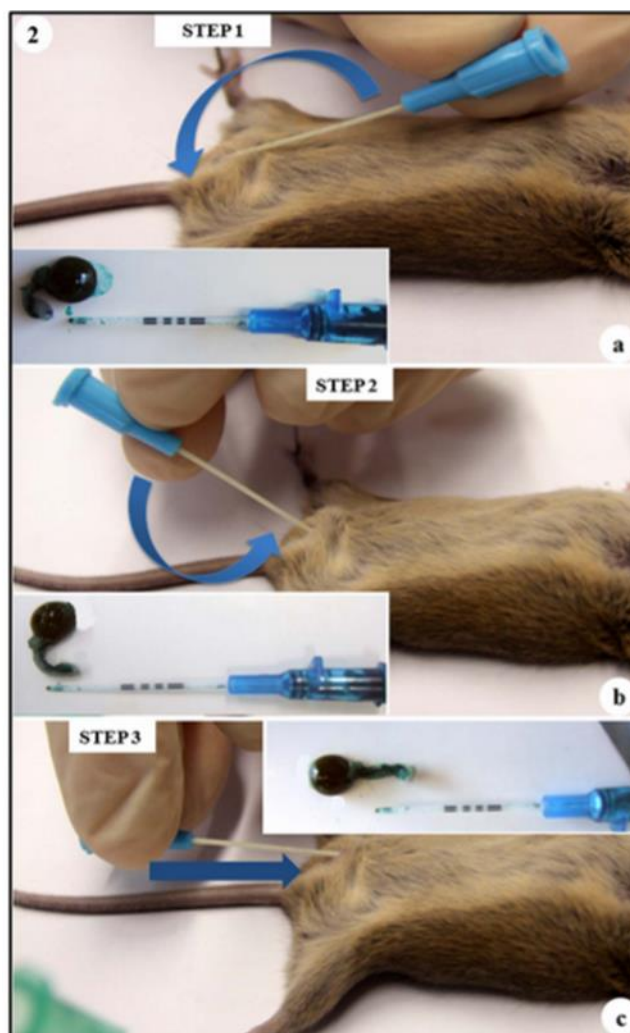
**Figure 5.1:** Area (cm<sup>2</sup>) of known volume of mouse urine used to determine voided volume from mice during VPA

## TREATMENTS AND SCHEDULES

Bladder cancer chemotherapeutic drugs were instilled into the bladder by the intravesical route. Dr Glen Ulett provided technical assistance in intravesical treatments. Mice were anaesthetised with 1-3% isoflurane gas. Isoflurane is a potent anaesthetic that produces rapid induction and recovery, with easy control of the depth of anaesthesia. It is considered safe and effective for laboratory mice use (Szczesny et al., 2004). Isoflurane has also been shown to abolish the micturition reflex while being administered, which is an advantage during intravesical treatments (Matsuura and Downie, 2000).

Prior to bladder catheterisation, micturition was induced by mild abdominal massage to lubricate the urethra and to prevent dilution of solutions. Bladder catheterisation was performed with sterile intravenous 22x1 gauge catheters (Terumo, Japan) through the urethra. This technique was based on that reported by Reis et al. (2011). Briefly, under anaesthesia, mice were positioned in dorsal recumbence and the external urethral ostium was identified (**Figure 5.2a**).

The catheter was introduced to the external urethral ostium by approximately 3mm from a cranial to caudal direction, before a 180° circular caudal movement was completed, with the proximal tip of the catheter remaining static (**Figure 5.2b**). The catheter was then inserted approximately 7mm further into the urinary bladder (**Figure 5.2c**).



**Figure 5.2: Urinary bladder catheterisation in mice. A – Catheter tip inserted 3mm in the external urethral ostium, inset picture: representative position of urinary bladder and female urethra after catheter insertion. B – Alignment of catheter with female urethra, inset picture: representative position of the catheter in urethra. C – Catheter introduction to the bladder, inset picture: representative position of the urinary bladder and female urethra after complete catheter insertion in the bladder. Arrows represent the movement of the catheter in the urethra and urinary bladder catheterization steps (Reis et al., 2011).**

Approximately 30µL of solution was instilled through the catheter, before removal. Mice were allowed to run freely in their cage with access to food, but not water, for 1 hour on Whatmann Filter Paper no. 1, ensuring they did not void during this treatment period. Micturition was induced 1 hour post-intravesical instillation to expel the drug or saline solution.

Animals were split into four treatment groups as per **Table 5.2**, treated with 30µL of gemcitabine (40mg/mL) dissolved in isotonic (0.9%) saline or isotonic saline alone, with voiding pattern analysis (VPA) performed directly prior to both intravesical treatment and euthanasia.

**Table 5.2: Voiding pattern analysis and treatment schedule for single and repeated saline and gemcitabine treated animal groups.**

	Single Saline Treatment	Single Gemcitabine Treatment	Repeated Saline Treatment	Repeated Gemcitabine Treatment
<b>Day 0</b>	<b>VPA analysis</b> 1x intravesical injection of isotonic (0.9%) saline, duration 1hr	<b>VPA analysis</b> 1x intravesical injection of 40mg/mL gemcitabine, duration 1hr	<b>VPA analysis</b> 1x (first) intravesical injection of 0.9% saline, duration 1hr	<b>VPA analysis</b> 1x (first) intravesical injection of 40mg/mL gemcitabine, duration 1hr
<b>Day 1 (+24hrs from Day 0)</b>	<b>VPA analysis</b> Animals euthanized for experiments	<b>VPA analysis</b> Animals euthanized for experiments	---	---
<b>Day 7</b>	---	---	1x (second) intravesical injection of 0.9% saline, duration 1hr	1x (second) intravesical injection of 40mg/mL gemcitabine, duration 1hr
<b>Day 8 (+24hrs from Day 7)</b>	---	---	<b>VPA analysis</b> Animals euthanized for experiments	<b>VPA analysis</b> Animals euthanized for experiments
<b>Number of Animals</b>	<b>6</b>	<b>6</b>	<b>5</b>	<b>5</b>

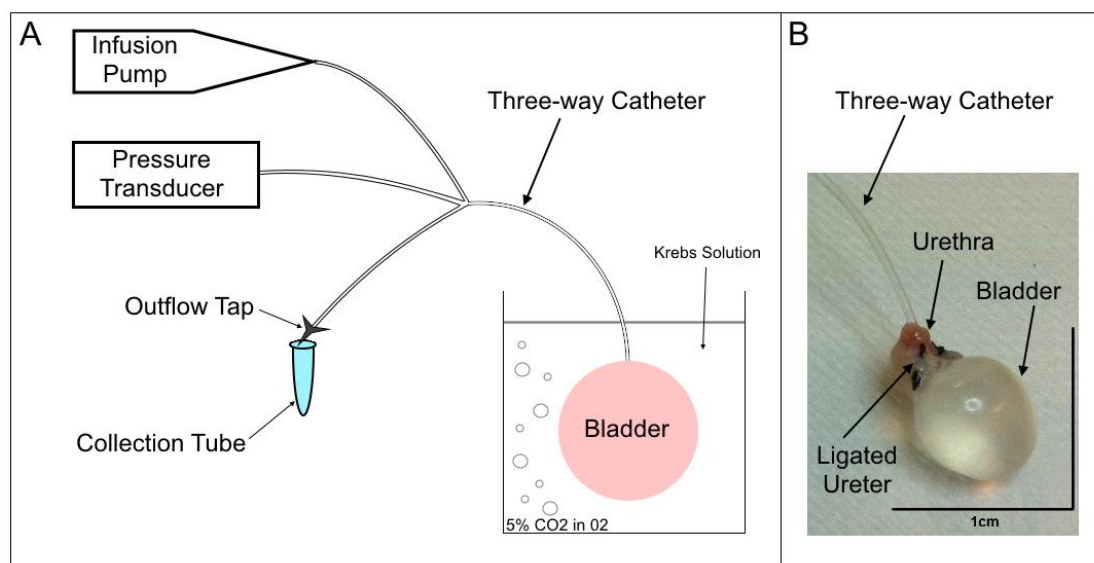
Animals were returned to their original cages and monitored 12 hours post treatment for signs of distress or pain. Sacrifice of mice was humanely performed either 24hrs or 8 days post initial treatment by cervical dislocation in accordance with ethical committee approval and the guidelines of the National Health and Medical Research Council of Australia. An incision was

made in the abdomen of the mouse, removing the skin and peritoneum of the abdomen. The gastrointestinal tract superior to the bladder was removed, revealing the kidneys. The vertebrae of the animals were severed above the kidneys at approximately the level of L2, and the tail and hind limbs removed. The remaining pelvic area was placed into ice-cold Krebs-bicarbonate solution for transport from the Griffith University animal facility to the research labs at Bond University.

## **ISOLATED BLADDER SET UP**

After transport to the Bond University laboratory, the pelvis area of the sacrificed mouse was secured in a 30mL bath, continually superfused with Krebs-bicarbonate solution (6mL/min) gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, maintained at a constant temperature of 37°C. Using a dissection microscope (WPI, PZMIII), the ovaries, the superior portion of the uterus, fallopian tubes and surrounding connective tissue were dissected away from the bladder, exposing the pubic symphysis. The ureters were ligated with silk suture proximal to the bladder to prevent leakage of intraluminal fluid. The pubic symphysis was cut either side of the urethra and removed, along with the pelvic bone and remaining portions of the hind legs. The urethra was identified, and carefully dissected from the remaining uterus and vagina of the mouse. A three-way catheter was inserted through the urethra into the bladder, and secured using silk sutures. The bladder was then dissected from the remaining pelvic area, with ureters cut above the level of the ligation. The isolated bladder was immediately placed in a 10mL organ bath containing 8mL Krebs-bicarbonate solution at 37°C, gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

The ends of the catheter inserted through the urethra to the bladder were attached respectively to an infusion pump to fill the bladder, another to a two-way outflow tap to allow emptying of the bladder and the third to a pressure transducer to measure intravesical pressure, shown by **Figure 5.3**.



**Figure 5.3: A - Schematic of the isolated mouse bladder set up, depicting the bladder connected to the three-way catheter. B - Photograph of a mouse bladder connected to a three-way catheter.**

The pressure transducer was attached to a Neurolog headstage (NL100, Digitmer Ltd, UK) connected to a pressure amplifier (NL 108, Digitmer Ltd, UK) which amplified the pressure signals before they were passed to a Micro1401 analogue to digital interface (Cambridge Electronic Design, UK) and then visualised on a computer using Spike2 software (version 7.1, Cambridge Electronic Design, UK).

The bladder was distended by infusing isotonic saline (0.9%) solution at 30 $\mu$ L/min to a maximum pressure of 40mmHg to check the viability of the catheterisation (leakages). The infusion pump was stopped and the outflow tap opened to allow passive removal of the intraluminal contents of the bladder. Baseline pressure from the emptied bladder in the closed system was subtracted from the pressures during distensions and agonist/nerve-mediated responses to give the change in intraluminal pressure. The isolated bladder was washed with fresh Krebs bicarbonate solution, distended to 25mmHg and allowed to stabilize for ~1 hour.

## **BLADDER COMPLIANCE AND SPONTANEOUS PHASIC CONTRACTIONS**

The effect of intravesical gemcitabine pre-treatment on the compliance of the bladder was examined by plotting bladder volume vs pressure curves. Additionally, phasic intravesical pressure increases were observed during filling of the bladder. These spontaneous contractions were similarly observed during the accommodation period after distension of the bladder to 25mmHg. The frequency and amplitude of spontaneous contractions during filling were measured over a 100s period directly following intraluminal pressure exceeding 20mmHg. During accommodation, the frequency and amplitude of spontaneous contractions was measured toward the end of the 1hr stabilization period.

## **MEASUREMENT OF UROTHELIAL MEDIATORS**

After confirmation of the viability of the bladder system, samples of serosal and intraluminal contents were collected for analysis of urothelial mediator release. After bladder emptying, the outflow tap was closed and the bath was washed and replaced with 8mL of fresh Krebs-bicarbonate solution. The infusion pump was turned on to fill the bladder with isotonic saline at a rate of 60µl/min, to a pressure of 25mmHg. Once the pressure reached 25mmHg, infusion was stopped and the outflow tap was opened. The luminal contents of the bladder were collected in a tube on ice and were stored at -20°C as soon as possible. A sample of the Krebs solution surrounding the bladder was taken for examination of release of mediators from the serosal tissue, also stored at -20°C for later analysis. This process was repeated after washing with fresh Krebs-bicarbonate solution. The luminal and serosal samples were later analysed for the release of ATP, acetylcholine and prostaglandin E<sub>2</sub> using commercially available kits described in Chapter 2.

## **RESPONSE TO PHARMACOLOGICAL AGENTS**

To investigate the effects of gemcitabine treatment on receptor-mediated muscle tension, the isolated bladder was filled to a set pressure of 25mmHg and allowed to stabilise for 1 hour. Contraction of the detrusor was instigated with the addition of the muscarinic agonist carbachol

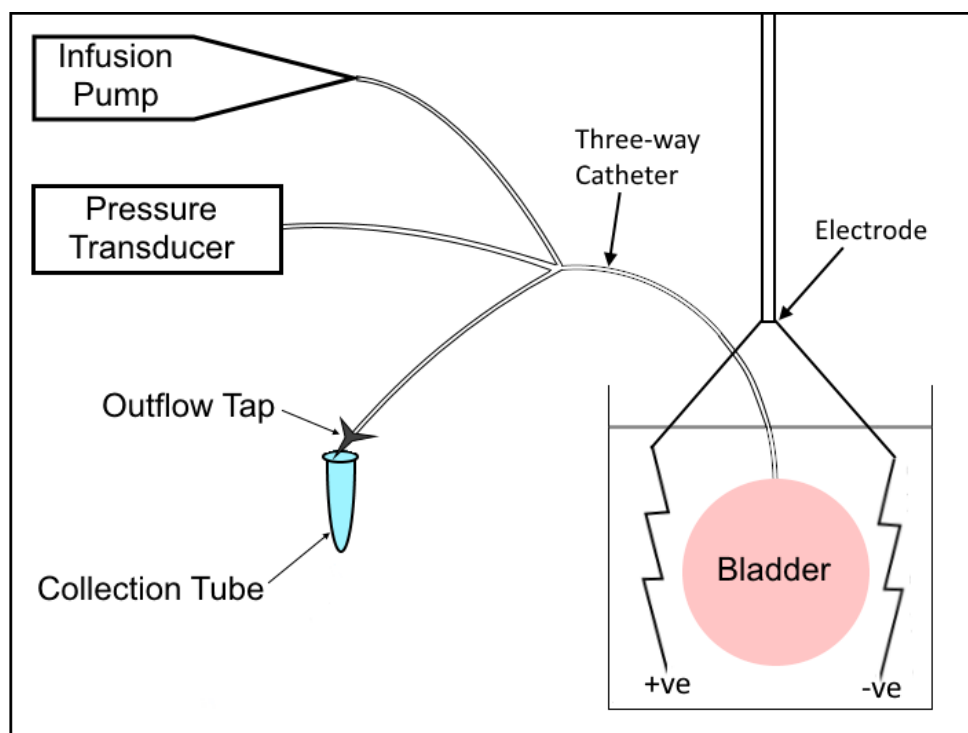


(10 $\mu$ M) to the bath. After emptying and washing with fresh Krebs-bicarbonate, the bladder was re-filled to 25mmHg pressure and allowed to re-stabilize. Contraction to carbachol was repeated in the presence of the competitive inhibitor of nitric oxide synthase (NOS) L-N<sup>G</sup>-Nitroarginine (L-NNA, 10 $\mu$ M).

To investigate the effect of gemcitabine on the  $\beta$ -adrenoceptor mediated relaxation of the bladder, the non-selective  $\beta$ -adrenoceptor agonist isoprenaline was used. After washing, filling to 25mmHg and stabilization, cumulative additions of isoprenaline were added to the bath (100ng - 100 $\mu$ g), and the effect of muscle tension recorded as change from baseline.

## **ELECTRICAL FIELD STIMULATION**

As contraction of the bladder is mediated primarily via the efferent nerves, the effect of intravesical gemcitabine treatment in mice on bladder contractions induced via nerve stimulation was investigated. After washing, the bladder was filled to 25mmHg and allowed to stabilise. The effect of electrical field stimulation was induced by placing platinum electrodes either side of the bladder (**Figure 5.4**). The bladder was electrically stimulated (50V, 0.1ms delay, 0.2ms pulse duration), delivered as a 5-second train every 100 seconds, at frequencies 1, 5, 10 and 20Hz. The tissues were stimulated at each frequency until stable responses were obtained, generally 4 spikes. Contraction strength was recorded as change in pressure from baseline.



**Figure 5.4: Schematic diagram of the isolated mouse bladder and electrical field stimulation set up. The bladder was filled through the infusion pump, stimulated by the current passing through the positive and negative electrodes, recorded by the pressure transducer.**

The contribution of various receptors and mediators on the nerve-mediated contraction in gemcitabine pre-treated bladders was investigated. Electrical field stimulation was repeated in the presence (15 minute pre-incubation) of 10 $\mu$ M L-NNA (NOS inhibitor), 1 $\mu$ M atropine (muscarinic antagonist) and after desensitization of P2X receptors with 10 $\mu$ M  $\alpha,\beta$ -mATP. Additionally, as  $\alpha,\beta$ -mATP is a competitive agonist of the P2X purinoceptors, the tension developed through the addition of this drug was recorded. The nerve-mediated contraction of the bladder to EFS was confirmed during preliminary experiments through the addition of the neurotoxin tetrodotoxin (1 $\mu$ M), which abolished the contractile response to the EFS parameters used within this study.

Finally, 60mM KCl was added to the system to measure the non-receptor mediated contractile response of the bladder tissue.

## **HISTOLOGY**

Whole isolated mouse bladders were fixed in 10% neutral buffered formalin for 24 hours at 4°C for histology. A catheter attached to a syringe containing formalin was inserted into the bladder through the urethra and secured using silk suture. The bladder was filled with formalin using the syringe and submerged in a beaker of formalin, fixing the tissue in a partially distended state. After 24 hours, the bladder was cut in two, before processing, sectioning and staining as described in Chapter 2.

## 5.4 RESULTS

### GENERAL EFFECTS OF TREATMENT ON ANIMALS

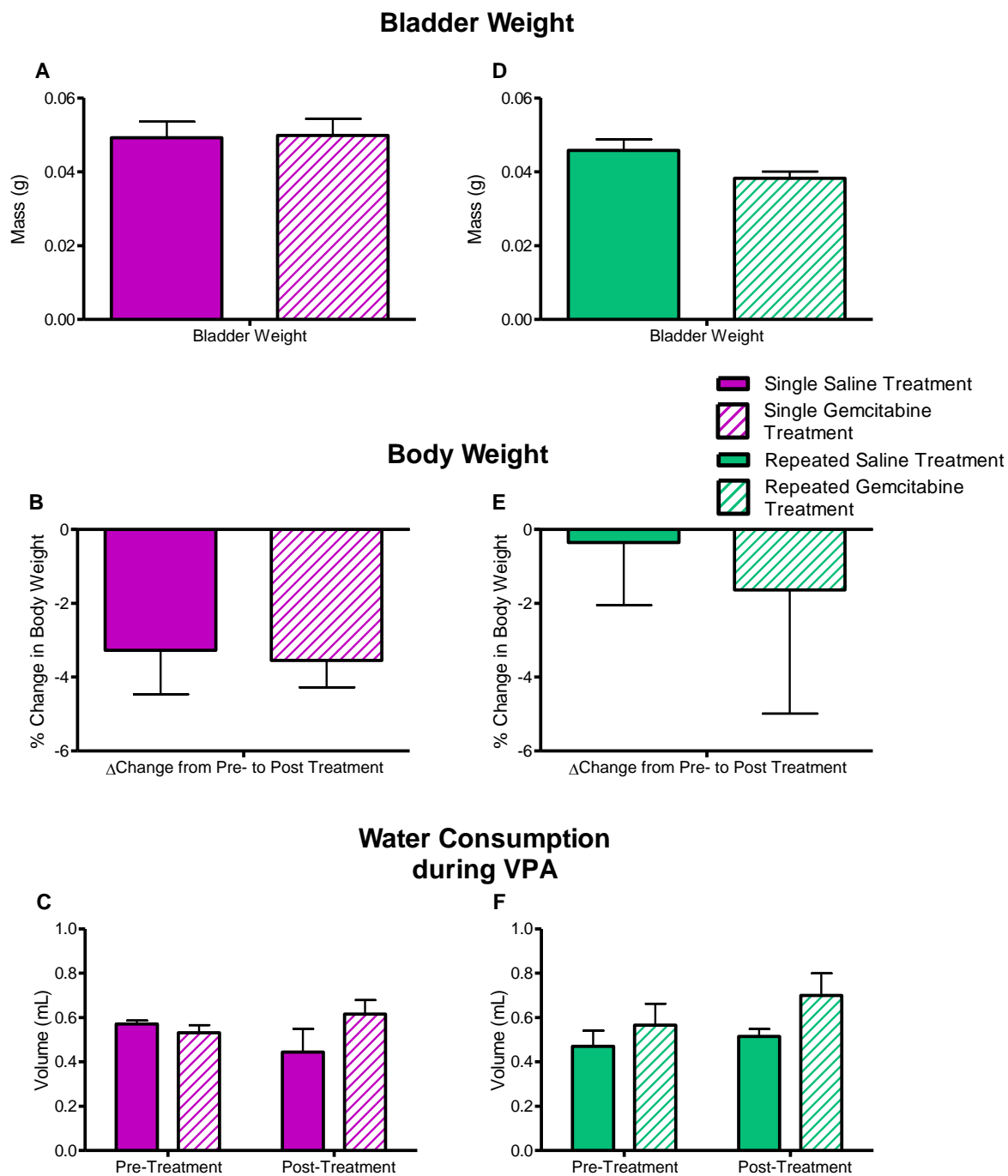
Prior to intravesical treatment, there was no significant difference in body mass between animals allocated to each treatment group. On average, animals from all treatment groups experienced loss in body mass after intravesical injections; however there was no statistically significant difference between them (**Table 5.3 & 5.4, Figure 5.5 B&E**). Similarly, the weights of the isolated bladders were unchanged between intravesical treatment groups (**Table 5.3 & 5.4, Figure 5.5 A&D**). Water consumption during the 4-hour voiding pattern analysis was recorded, and while the mice from the single and repeated gemcitabine treatment groups on average consumed more water post-treatment (0.09mL and 0.13mL respectively), this was not significantly different from the saline-treated animals (**Figure 5.5 C&F**).

**Table 5.3: Water consumption, body weight and bladder weight prior to and 24hrs after single intravesical treatment with saline or gemcitabine**

	Single Saline Treatment		Single Gemcitabine Treatment	
	Pre-Treatment	Post-Treatment	Pre-Treatment	Post-Treatment
<b>Water Consumption (mL)</b>	0.57±0.2	0.53±0.07	0.53±0.03	0.62±0.06
<b>Body Weight (g)</b>	27.29±1.01	26.35±0.75	28.86±0.38	27.85±0.52
<b>Bladder Weight (g)</b>		0.05±0.004		0.05±0.005

**Table 5.4: Water consumption, body weight and bladder weight prior to and post repeated intravesical treatment with saline or gemcitabine**

	Repeated Saline Treatment		Repeated Treatment	Gemcitabine
	Pre-Treatment	Post-Treatment	Pre-Treatment	Post-Treatment
<b>Water Consumption (mL)</b>	0.47±0.07	0.52±0.03	0.57±0.1	0.7±0.1
<b>Body Weight (g)</b>	27.08±0.73	26.35±0.46	28.55±0.86	27.94±1.29
<b>Bladder Weight (g)</b>		0.05±0.003		0.04±0.002



**Table 5.5: Effects of intravesical gemcitabine on bladder weight (A), body weight (B – measured as change from pre- to post-treatment) and water consumption during voiding pattern analysis. Data represented as mean  $\pm$  SEM (n $\geq$ 4).**

## VOIDING PATTERN ANALYSIS

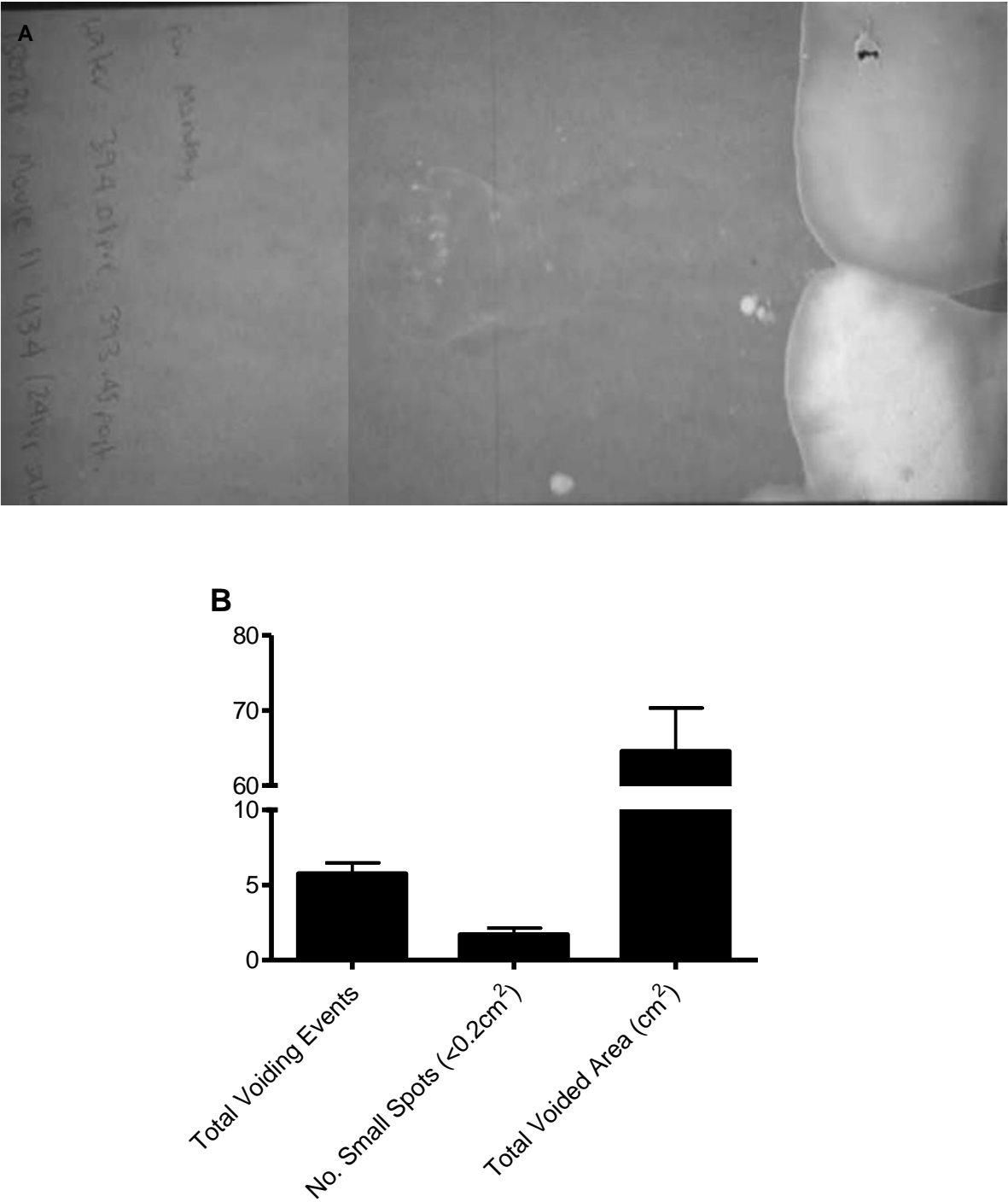
The voiding behaviour of mice treated intravesically was assessed by measuring and quantifying the urine output of freely moving mice during a four hour period. Voiding pattern analysis was performed before and after (24 hours) intravesical treatments. **Figure 5.6A** is a representative image of a photographed sheet of Whatmann Filter Paper 1 under UV light, with urine spots shown as fluorescent. Spots were defined as small if the area was measured to be less than  $0.2\text{cm}^2$ .

On average, mice urinated  $5.7 \pm 0.7$  times in a four hour period, with  $1.7 \pm 0.4$  of those spots on the filter paper being small (less than  $0.2\text{cm}^2$ ), as shown in **Figure 5.6B**. The total volume of the voided area during the 4 hours period was on average  $394.6 \pm 35.4\mu\text{L}$ .

Representative images of the voiding patterns 24-hours post intravesical treatment with saline and gemcitabine are seen in **Figure 5.7 A&B** respectively. Single intravesical treatments increased the number of voided spots during the 4-hour voiding pattern analysis (**Figure 5.7C**). Intravesical treatment with saline increased the total voided spots by 1.4 fold, while intravesical gemcitabine (40mg/mL) significantly enhanced the number of voided urine spots in female mice by 3.3 fold. The increase in spot number was primarily due to an increase in small-voids (less than  $0.2\text{cm}^2$ ). As seen in **Figure 5.7D**, intravesical saline and gemcitabine enhanced small urine spot production 24 hours after treatment, by 3.4 and 8.8 fold respectively. This change was significant in the intravesical gemcitabine group ( $p < 0.001$ ). Although the number of voids increased after intravesical treatments, the total area of voided spots was unchanged from pre- to post-intravesical treatments with both agents (**Figure 5.7E**).

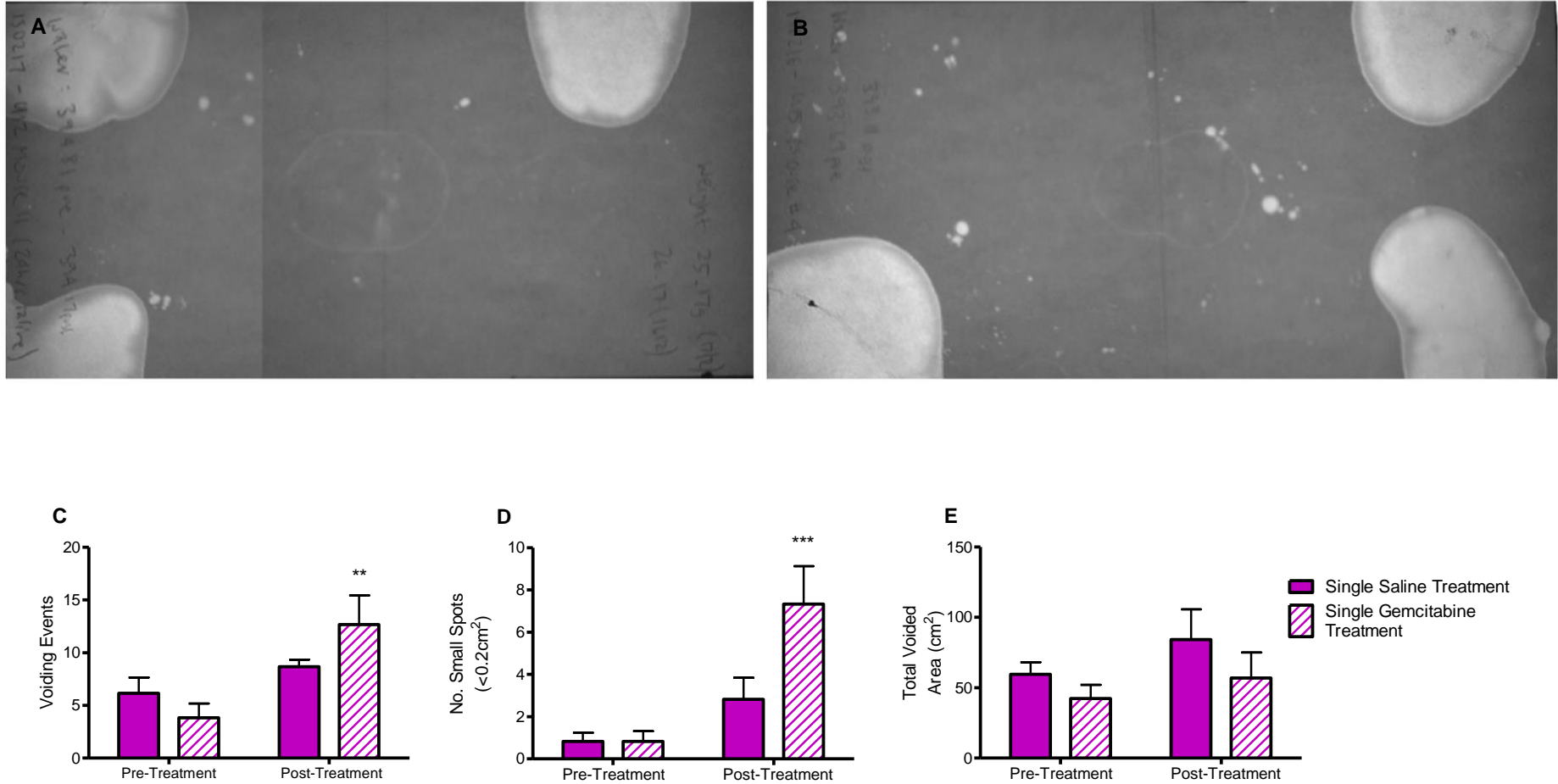
Voiding pattern analysis was also performed on mice undergoing repeated intravesical treatments with saline and gemcitabine (40mg/mL). Representative images of voided urine spots from animals 24 hours post repeated (two) treatments of saline and gemcitabine are shown in **Figure 5.8 A&B**. There was no change in the total number of voided spots pre- and post-intravesical treatments with saline, while repeated gemcitabine instillations significantly enhanced the number of voided spots, by 1.9 fold (**Figure 5.8C**). This increase in spot number was due to a 2.4-fold increase in small-voids (less than  $0.2\text{cm}^2$ ) after repeated treatments with gemcitabine (**Figure 5.8D**), similar to the results seen within the single intravesical gemcitabine

instillation mouse group. However, there was no change in the total voided area pre- and post-repeated intravesical treatments in either the gemcitabine or the saline groups (**Figure 5.8E**).

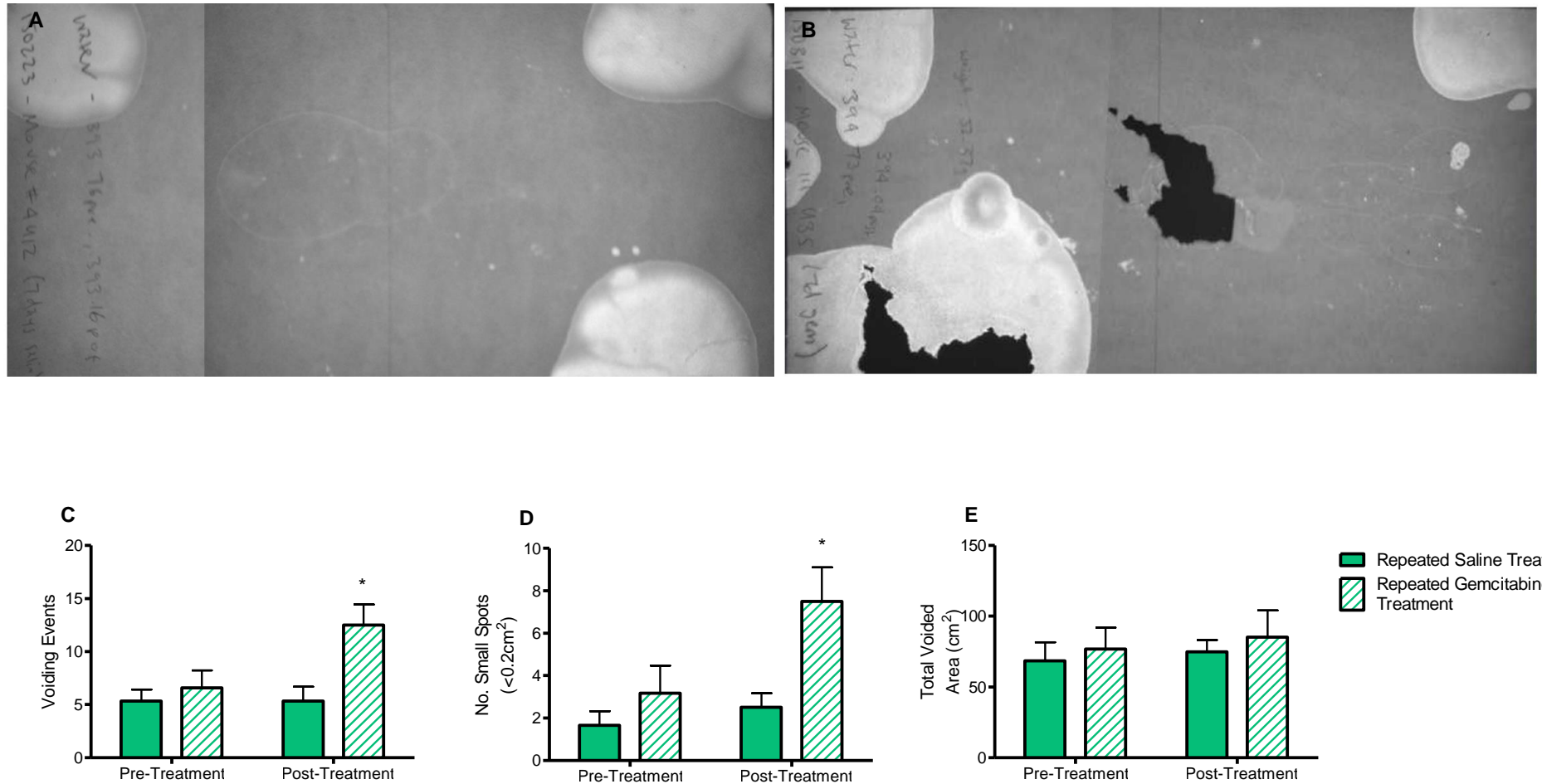


**Figure 5.6:** Representative images of voiding pattern analysis. Photographed sheet of Whatmann Filter Paper 1 showing urine spot patterns obtained from a mouse prior to intravesical treatment (A) over a 4-hour period. Urine spot patterns in mice prior to intravesical treatment (B). Data represented as mean ± SEM (n=23).





**Figure 5.7: Representative images of voiding pattern analysis.** Photographed sheets of Whatmann Filter Paper showing urine spots obtained from mice 24hours after a single dose of (A) intravesical isotonic saline and (B) intravesical gemcitabine (40mg/mL). The total number of voiding events (C), number of small spots (D) and total voided area (E) were measured pre- and post- intravesical treatment with gemcitabine or isotonic saline solution. Data is represented as mean  $\pm$  SEM (n=6), analysed using a one-way ANOVA with Bonferroni multiple comparisons post-hoc test (\*\*p<0.01, \*\*\*p<0.001 vs. pre-treatment)



**Figure 5.8: Representative images of voiding pattern analysis. Photographed sheets of Whatmann Filter Paper showing urine spots obtained from mice after repeated doses of (A) intravesical isotonic saline and (B) intravesical gemcitabine (40mg/mL). The total number of voiding events (C), number of small spots (D) and total voided area (E) were measured pre- and post- intravesical treatment with gemcitabine or isotonic saline solution. Data is represented as mean  $\pm$  SEM (n=5), analysed using a one-way ANOVA with Bonferroni multiple comparisons post-hoc test (\* $p$ <0.05 vs. pre-treatment)**

## HISTOLOGY

The effects of single and repeated intravesical instillations of gemcitabine (40mg/mL) and saline (0.9%) on the urothelium was observed through histological sections of the murine bladder. The width of the urothelium was unchanged between the single and repeated instillations of saline, however thickening of the urothelium was observed in the single and repeated gemcitabine-treated mice bladders (**Figure 5.9**).

Typical histological features of murine bladders, including an intact urothelium sitting atop the lamina propria/suburothelial and detrusor muscle layers, were observed in the saline treated mice (**Figure 5.10 A&B, E&F**). Slight oedema within the lamina propria/suburothelial layer resulting in detachment of urothelial cells from the detrusor muscle can be seen within the histological sections from mouse bladders treated with single and repeated intravesical instillations of gemcitabine (**Figure 5.10 C&D, G&H** respectively). Additionally, macroscopic signs of urinary bladder inflammation including redness and increased visible vasculature were observed in 3 and 4 (out of 6) bladders from single and repeated gemcitabine treated mice respectively.

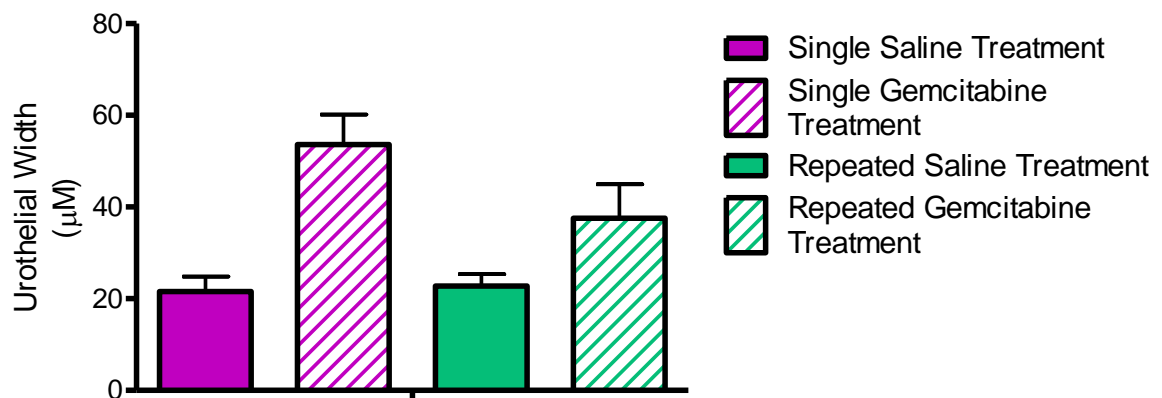
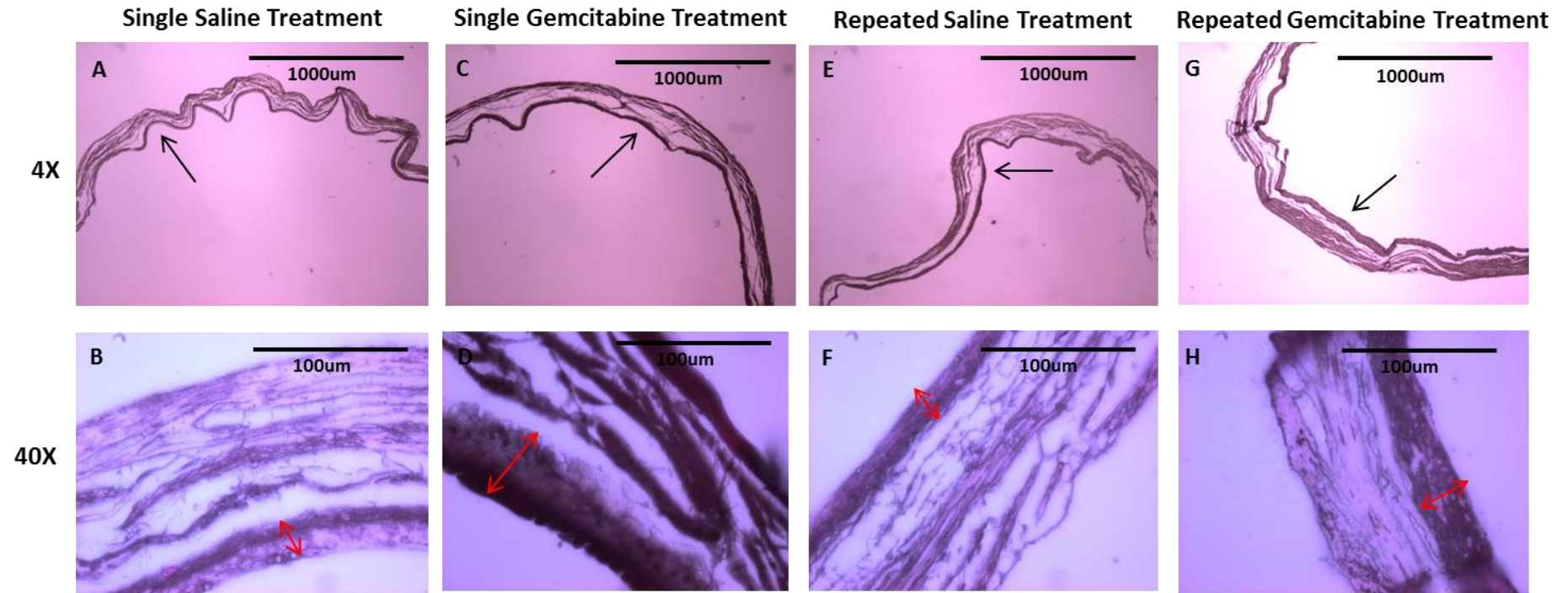


Figure 5.9: Urothelial thickness as determined by analysis with Image J is representative of n=2 results.



**Figure 5.10:** Histological sections of isolated mouse bladders stained with haematoxylin and eosin from single treatments with saline (A&B) or gemcitabine (C&D), and repeated treatments with saline (E&F) or gemcitabine (G&H) at 4X and 40X respectively. Arrows indicate the width of the urothelium.

## MEDIATOR RELEASE

Samples of intraluminal and serosal fluid were collected from bladders distended to 25mmHg and analysed for release of mediators ATP, acetylcholine and prostaglandin E<sub>2</sub>. Two distensions were performed consecutively with approximately 10 minutes recovery between, to measure the repeatability of mediator release in response to stretch. There was no change in the release of ATP, acetylcholine or prostaglandin E<sub>2</sub> between distensions from either the luminal or serosal sides (**Figure 5.11 A-C**).

As the volumes of fluid in the lumen and surrounding serosa were different (<0.5mL and 8mL respectively), the mediator concentrations were normalised to volume to measure total amount released by the respective sides of the bladder tissue. The serosal fluid contained more than 30-fold the amount of ATP (pmoles) than the luminal fluid ( $p<0.001$ ) (**Figure 5.11A**). Similarly, the release of acetylcholine and prostaglandin E<sub>2</sub> was significantly greater from the serosal samples compared to the luminal release, by approximately 34- and 28- fold respectively ( $p<0.001$ ,  $p<0.05$ ) (**Figure 5.11 B&C**).

The effect of single and repeated intravesical treatments with saline and gemcitabine on mediator release from mouse bladders was investigated. The release of ATP into the lumen of the bladder was enhanced 2.8-fold following repeated instillations of intravesical gemcitabine compared to controls (**Figure 5.12A**). Furthermore, repeated gemcitabine treatments increased luminal ATP release by 370% ( $p<0.001$ ) compared to a single gemcitabine treatment (**Figure 5.12A**). No change was observed in ATP release from the serosal side between treatment groups (**Figure 5.12D**). Similarly, the release of acetylcholine from the bladder into the luminal and serosal fluid was unchanged with single or repeated intravesical instillations of gemcitabine (40mg/mL) (**Figure 5.12 B&E**).

Repeated intravesical treatments with gemcitabine significantly enhanced release of prostaglandin E<sub>2</sub> ( $p<0.05$ ) compared to controls by 4.6- and 3.6-fold into the luminal and serosal fluid respectively (**Figure 5.12 C&F**). Furthermore, repeated instillations of gemcitabine enhanced prostaglandin E<sub>2</sub> release by 3.2- and 2.2-fold compared to a single instillation of gemcitabine into the luminal and serosal fluid respectively (**Figure 5.12 C&F**).

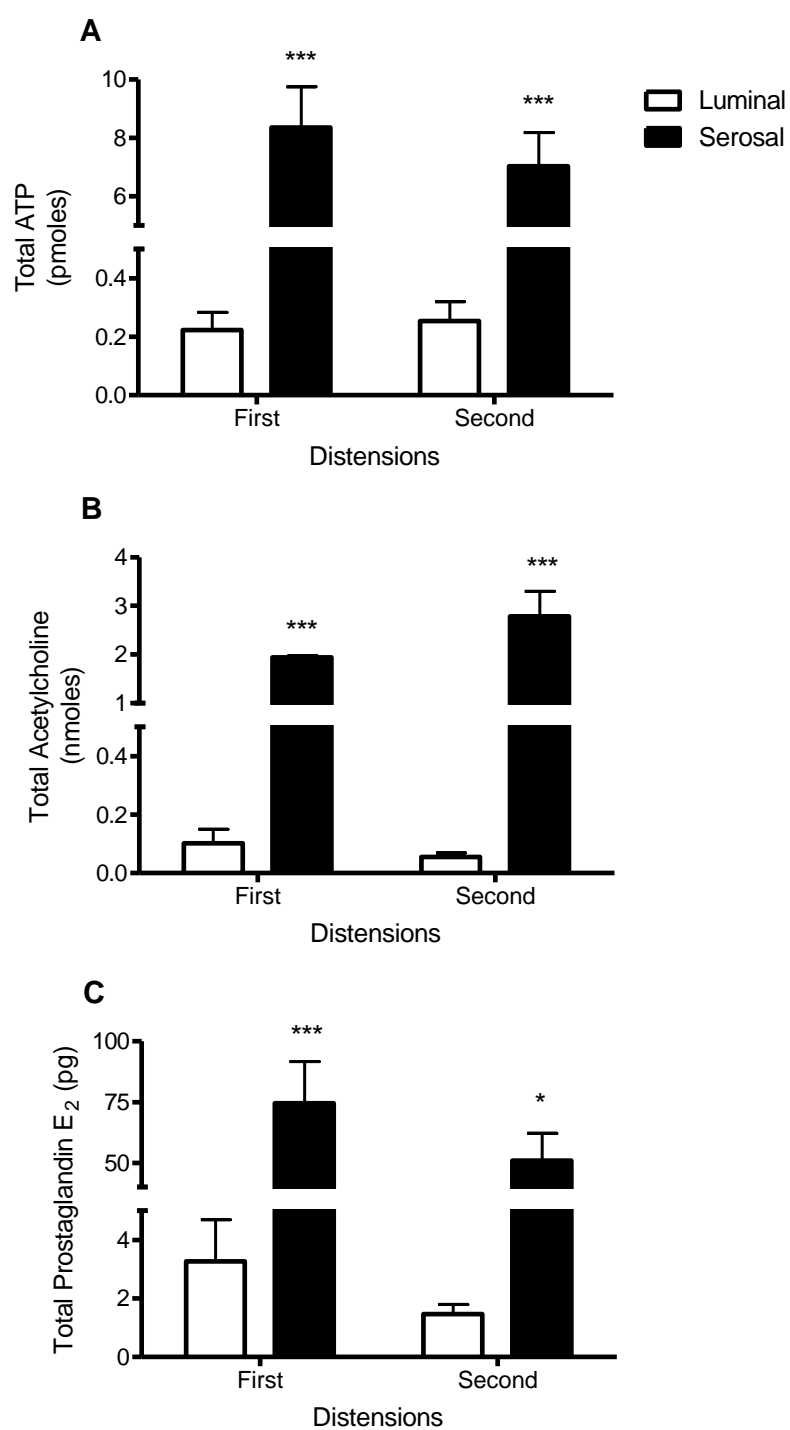


Figure 5.11: Repeatability of total ATP (A), acetylcholine (B) and prostaglandin E<sub>2</sub> (C) from consecutive luminal and serosal samples from stretched mouse bladders. Data represented as mean  $\pm$  SEM (n=6), analysed using a one-way ANOVA with Dunnetts multiple comparisons post-hoc test (\*p<0.05, \*\*\*p<0.001 vs luminal sample)

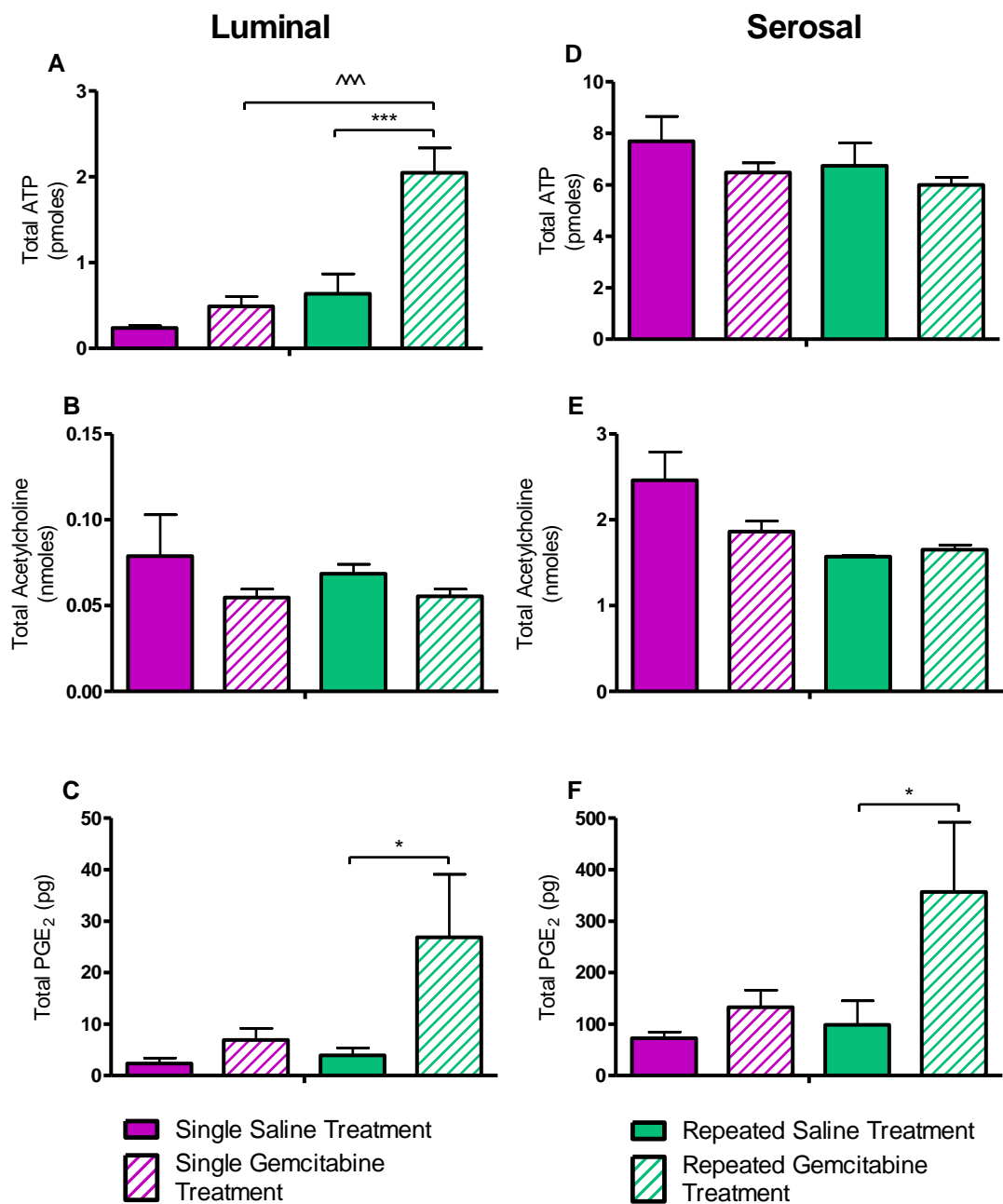
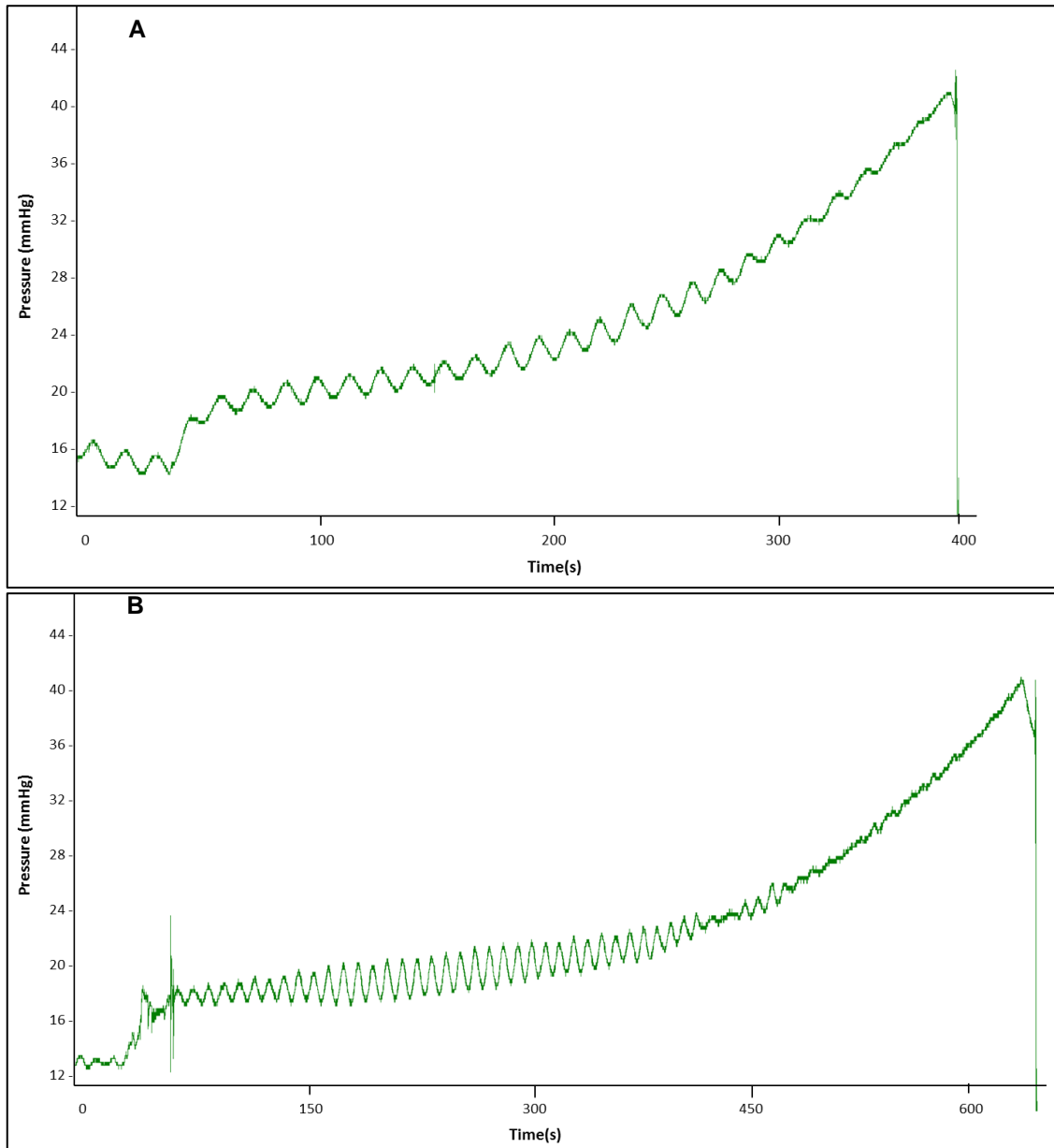


Figure 5.12: Effect of single and repeated intravesical gemcitabine treatments on the release of ATP (A&D), acetylcholine (B&E) and prostaglandin E<sub>2</sub> (C&F) from the luminal (A-C) and serosal (D-F) surfaces of the bladder. Data is represented as mean  $\pm$  SEM ( $n \geq 5$ ), normalised to volume, analysed using a one-way ANOVA with Bonferroni multiple comparisons post-hoc test (\* $p < 0.05$ , \*\*\* $p < 0.001$  vs repeated saline treatment group, ^^^ $p < 0.001$  vs single gemcitabine treatment group)

## SPONTANEOUS CONTRACTIONS

During bladder distension, spontaneous phasic contractions were observed in both saline and gemcitabine treated bladders, as shown in **Figure 5.13 A&B**. Furthermore, the contractions persisted during bladder accommodation to 25mmHg pressure. Single and repeated intravesical instillations of gemcitabine (40mg/mL) did not have any effect on the frequency and amplitude of the spontaneous contractions during both filling and accommodation (**Figure 5.14 A&B**)



**Figure 5.13: Representative traces showing full distension (to 40mmHg) of isolated bladders from mice undergoing single intravesical treatments with saline (A) and gemcitabine (40mg/mL) (B). Note the spontaneous phasic contractions during filling.**



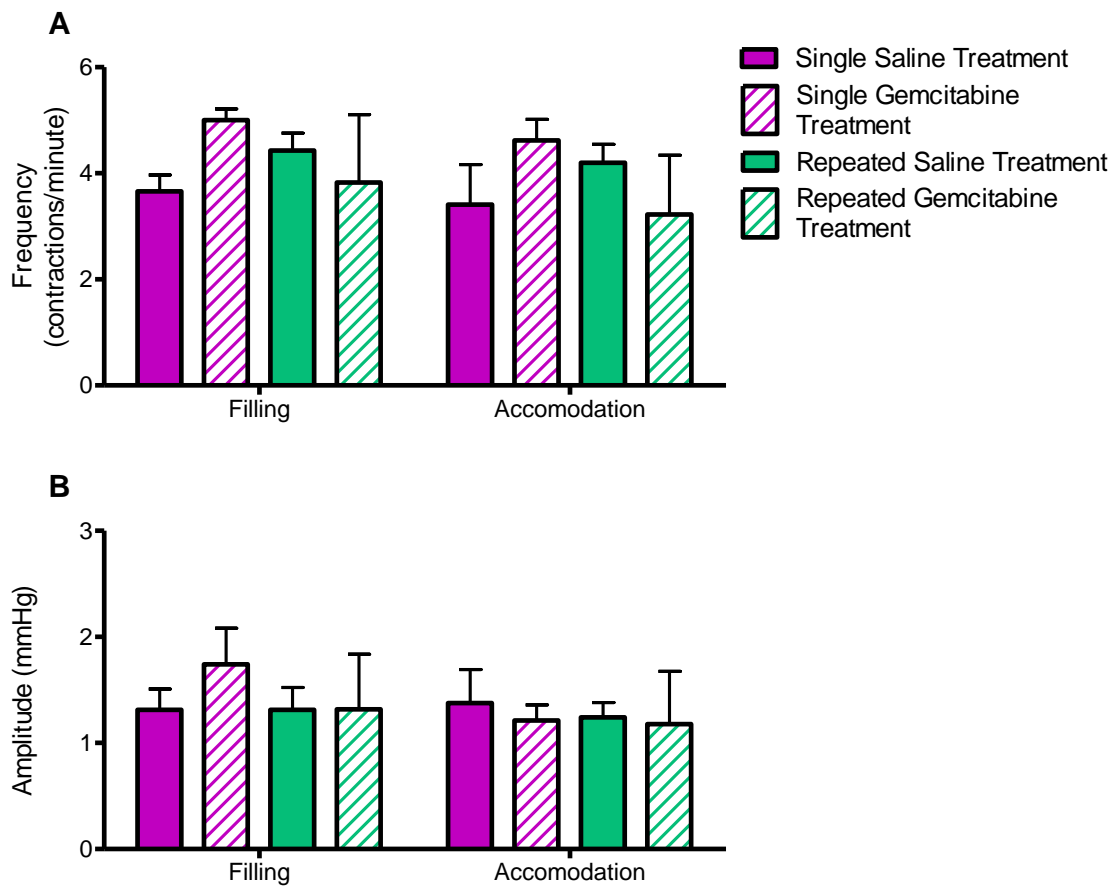
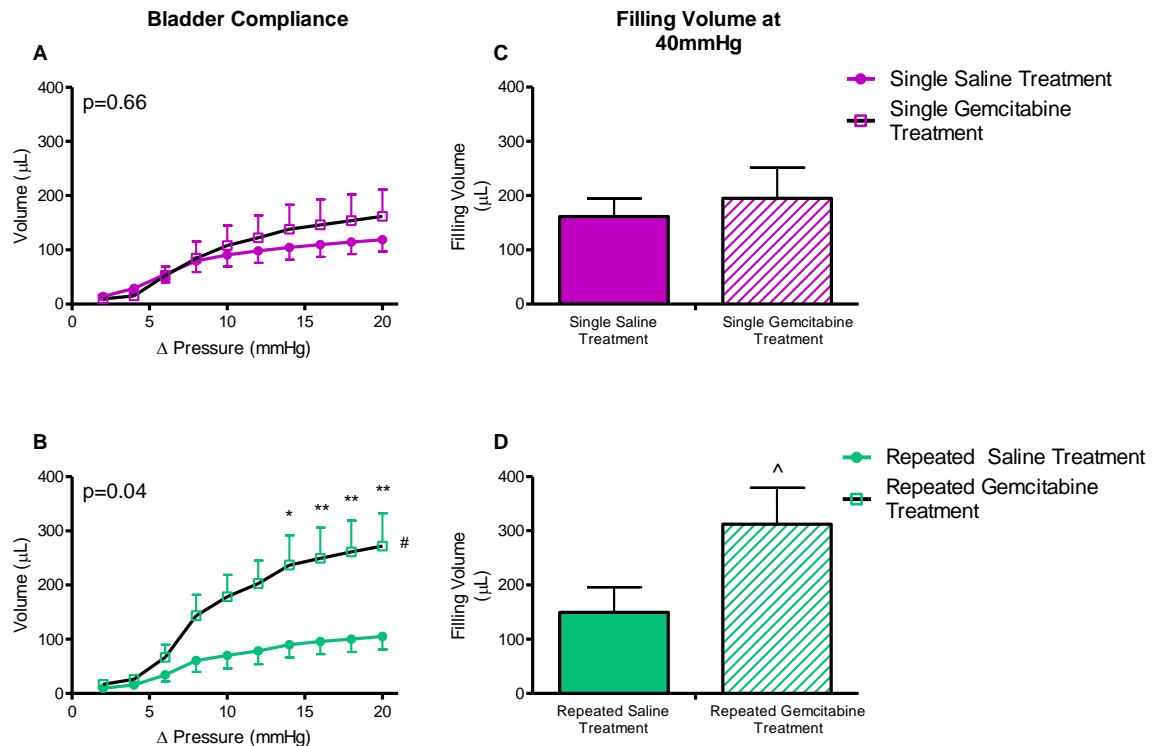


Figure 5.14: Frequency (A) and amplitude (B) of spontaneous contractions from bladders from single and repeated intravesical treatments with saline and gemcitabine (40mg/mL) during filling to 40mmHg and accommodation to 25mmHg pressure. Data measured from the 100s directly after intraluminal pressure reached 20mmHg, represented as mean  $\pm$  SEM ( $n \geq 4$ ), analysed by one-way ANOVA with Bonferroni multiple comparisons post-hoc test.

## BLADDER COMPLIANCE AND FILLING

Isolated bladders from mice were infused with isotonic saline to an intraluminal pressure of 40mmHg to investigate changes in muscle compliance. Intravesical pre-treatment with gemcitabine increased the compliance in murine bladders, significantly ( $p<0.05$ ) in the repeated treatment groups (**Figure 5.15 A&B**). The volume of bladders at 40mmHg intravesical pressure was greater in gemcitabine pre-treated groups with comparison to the matched saline controls. Single and repeated instillations of gemcitabine enhanced the filling volume by 20% and 109% respectively, the latter significantly increased ( $p<0.05$ ) (**Figure 5.15 C&D**).



**Figure 5.15: Volume-pressure curves (A&B) and filling volume at 40mmHg of bladders from single (A&C) and repeated (B&D) intravesical treatments with saline and gemcitabine (40mg/mL). Data represented as mean  $\pm$  SEM ( $n\geq 4$ ), analysed by two-way ANOVA with Bonferroni multiple comparisons post-hoc test (# $p<0.05$  gemcitabine treated curve vs saline treated curve, \* $p<0.05$  and \*\* $p<0.01$  vs saline treated at respective pressures) and an unpaired student t-test (^ $p<0.05$  vs repeated saline group).**

## RESPONSES TO PHARMACOLOGICAL AGENTS

Bladders were distended by filling with isotonic solution to an intravesical pressure of 25mmHg, and left for approximately 1 hour to accommodate the volume and muscle tension to stabilise. Pharmacological agents were added to the serosal fluid to measure changes in receptor-mediated contraction/relaxation. Single and repeated intravesical instillations of gemcitabine (40mg/mL) had no effect on subsequent *in vitro* contraction to carbachol, nor did the inhibition of NOS by L-NNA potentiate the contraction to carbachol between any of the treatment groups (**Figure 5.16A**). Similarly, there was no difference between contraction to P2X receptor stimulation (with  $\alpha,\beta$ -mATP) between the treatment groups (**Figure 5.16B**). Finally, muscle contraction induced by the addition of KCl was also unchanged between treatment groups in murine bladders.

The addition of the  $\beta$ -adrenoceptor agonist isoprenaline to the serosal fluid caused relaxation. Bladders treated with gemcitabine exhibited greater relaxation responses than the matched saline groups (>20% increase in single treatment group, >42% increase in repeated treatment group), but these were not statistically significant differences (**Figure 5.17 A&B**). Furthermore, the  $pIC_{50}$  values for each group were unchanged (saline vs gemcitabine pre-treatment,  $8.0 \pm 0.9$  vs  $8.0 \pm 1.7$  and  $8 \pm 1.1$  vs  $7.1 \pm 0.3$  in single and repeated groups respectively).

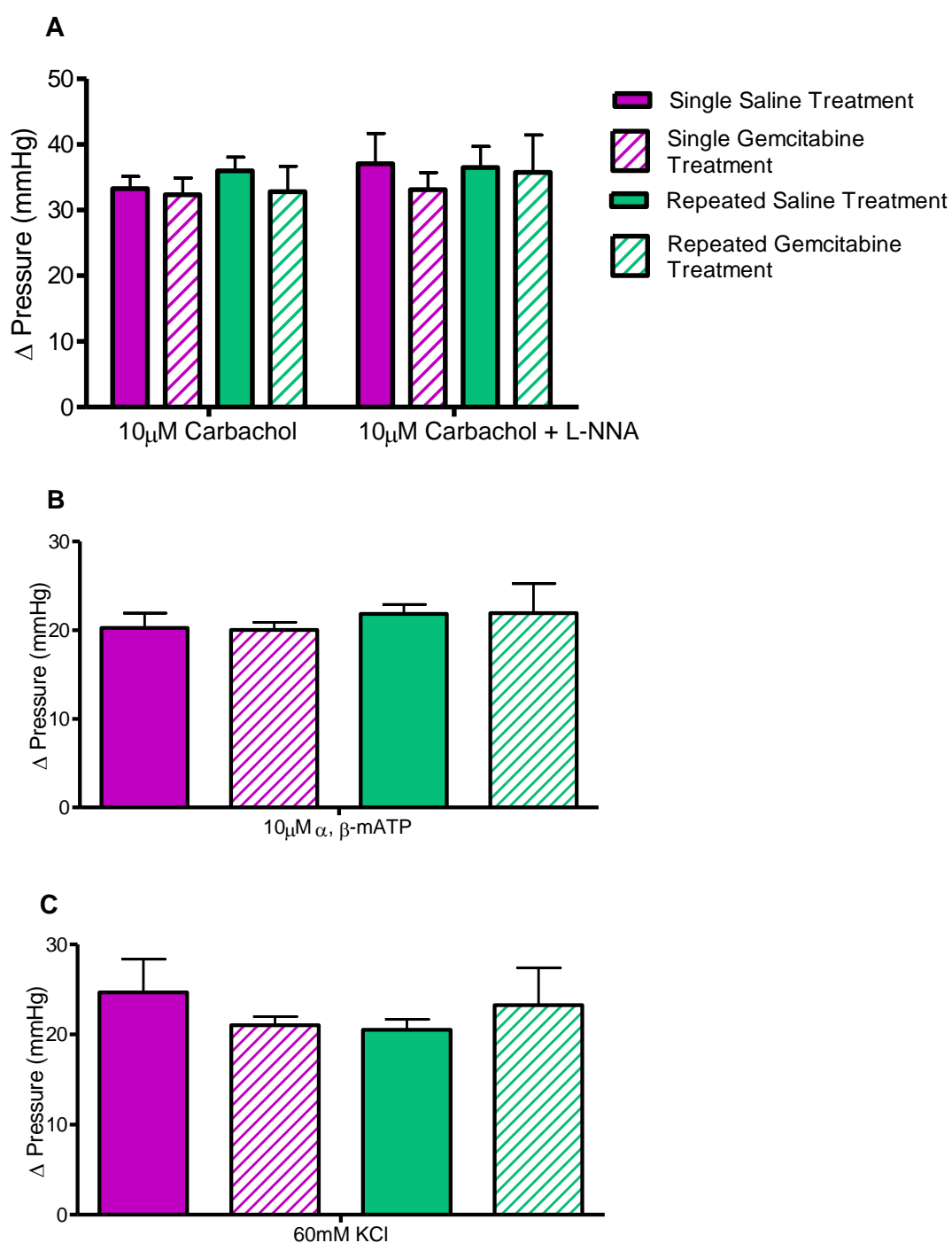
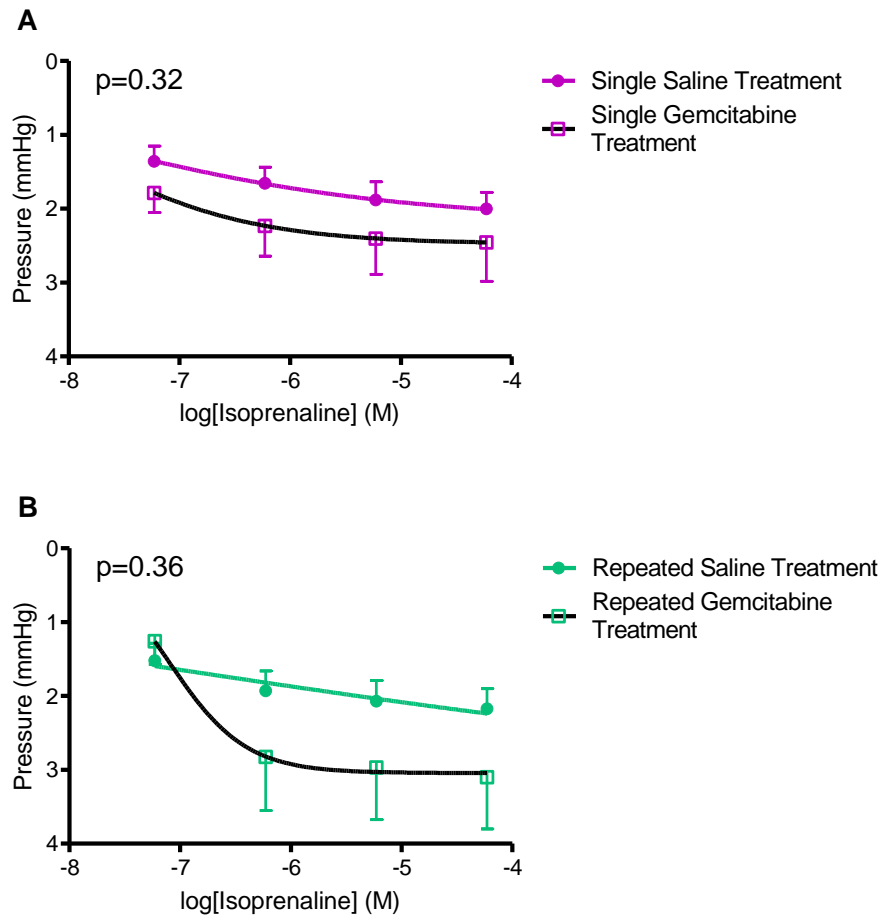


Figure 5.16: Effect of single and repeated intravesical instillations of saline (0.9%) or gemcitabine (40mg/mL) on the contraction of the bladder to carbachol without and in the presence of L-NNA (A),  $\alpha$ , $\beta$ -mATP (B) and KCl (C). Data represented as mean  $\pm$  SEM (n $\geq$ 4), analysed by one-way ANOVA with Bonferroni multiple comparisons post-hoc test.

## Relaxation to Isoprenaline



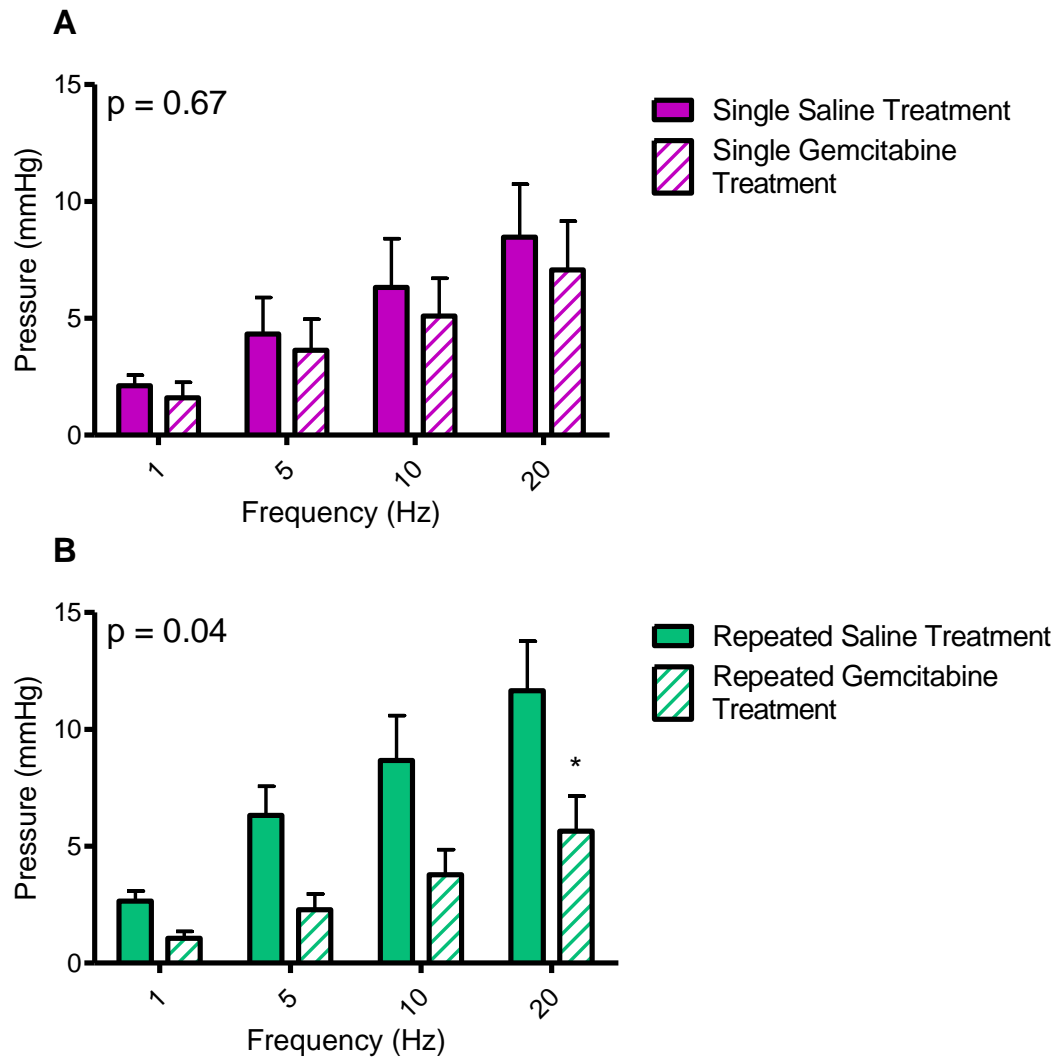
**Figure 5.17: Effect of single (A) and repeated (B) intravesical instillations of saline (0.9%) or gemcitabine (40mg/mL) on the relaxations of the bladder to isoprenaline. Data represented as mean  $\pm$  SEM ( $n \geq 5$ ), analysed by a two-way ANOVA with Bonferroni multiple comparisons post-hoc test.**

## CONTRACTION TO ELECTRICAL FIELD STIMULATION

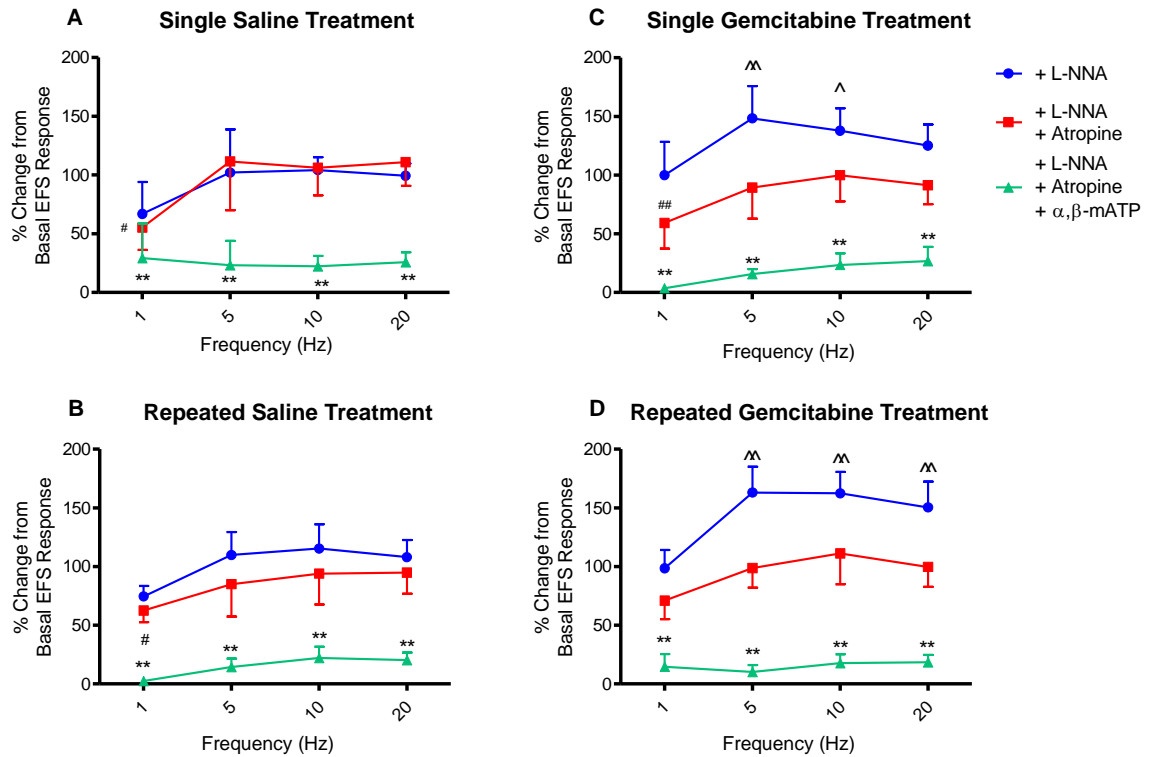
Electrical field stimulation (EFS) was used to investigate the effect of single and repeated instillations of gemcitabine (40mg/mL) on the efferent nerve-mediated contraction in mouse bladders. Pre-treatment with single or repeated instillations of intravesical gemcitabine depressed the contraction to EFS, although this was only significant in the repeated treatments group ( $p<0.05$ ) (**Figure 5.18 A&B**). At the 20Hz frequency, gemcitabine depressed the contraction by 18% and 52% with comparison to saline-instilled bladders in the single and repeated treatment groups respectively (**Figure 5.18 A&B**).

The addition of the pharmacological agents L-NNA, atropine and  $\alpha,\beta$ -mATP were added to the serosal fluid to investigate the respective contributions of nitric oxide, muscarinic and P2X receptors to nerve-mediated responses. In the single and repeated saline pre-treated bladders, the addition of L-NNA had no effect on contraction to EFS (**Figure 5.19 A&B**). The addition of atropine significantly inhibited ( $p<0.05$ ) contractions at low frequencies (1Hz) by 45% and 41% in the single and repeated groups respectively. Finally, the inhibition of the P2X receptors by the addition of  $\alpha,\beta$ -mATP significantly attenuated ( $p<0.01$ ) the contraction to EFS by >75% across all frequencies measured.

In bladders pre-treated with intravesical gemcitabine (40mg/mL), the addition of the NOS inhibitor L-NNA augmented contractions to EFS (**Figure 5.19 C&D**). In the single gemcitabine treatment group, contractions were significantly ( $p<0.05$ ) enhanced by 1.4- and 1.5-fold at 5Hz and 10Hz respectively (**Figure 5.19C**). Contractions to high frequency stimulation (>5Hz) in the repeated gemcitabine treatment group were increased by >1.5-fold (**Figure 5.19D**). Similar to the saline-treated bladders, the addition of atropine attenuated the contraction to low frequencies in the single gemcitabine-treated group, although no change was observed across any of the frequencies in the repeated gemcitabine-treatment group. Finally, the desensitization of P2X receptors with  $\alpha,\beta$ -mATP significantly ( $p<0.01$ ) reduced the contraction by a factor of 4 across all frequencies in both gemcitabine treatment groups.



**Figure 5.18: Effect of single (A) and repeated (B) intravesical instillations of saline (0.9%) or gemcitabine (40mg/mL) on the nerve-mediated contractions to EFS at 1, 5, 10 and 20Hz in isolated mouse bladders. Data represented as mean  $\pm$  SEM ( $n \geq 5$ ), analysed by a two-way ANOVA with Bonferroni multiple comparisons post-hoc test (\* $p < 0.05$  vs saline treated bladder at respective frequencies).**



**Figure 5.19: Effect of single and repeated intravesical instillations of saline (A&B respectively) (0.9%) or gemcitabine (C&D respectively) (40mg/mL) on the nerve-mediated contractions to EFS at 1, 5, 10 and 20Hz in isolated mouse bladders in the presence of L-NNA, atropine and  $\alpha, \beta$ -mATP.** Data represented as mean  $\pm$  SEM (n $\geq$ 4), analysed by a one-way ANOVA with Dunnett multiple comparisons post-hoc test, ( $^{\wedge}$ p<0.05,  $^{\wedge\wedge}$ p<0.01 L-NNA vs basal EFS response,  $^{\#}$ p<0.05,  $^{\#\#}$ p<0.01 atropine vs basal EFS response, \*p<0.05, \*\*p<0.01  $\alpha, \beta$ -mATP vs basal EFS response at respective frequencies.



## 5.5 DISCUSSION

Micturition is mediated by a complex coordination of afferent and efferent nerve activity, release of urothelial mediators and detrusor muscle tone. While the previous chapter investigated the changes in porcine bladder directly after luminal gemcitabine treatment, the present study aimed to investigate the physiological and functional effect of single and repeated intravesical gemcitabine instillations *in vivo*. Treatment parameters for the mouse groups mimicked the clinical setting, with instillations of 40mg/mL gemcitabine or the vehicle 0.9% saline given on a weekly schedule.

### GENERAL EFFECT OF TREATMENT ON ANIMALS

Single and repeated instillations of gemcitabine appeared to have no effect on the general well-being of mice. Animals were inspected daily for signs of distress, with no observable differences in behaviour or appearance, and there was no difference in water consumption during the VPA period between the saline and gemcitabine group. Neither saline nor gemcitabine treated animals underwent any significant weight loss over the treatment period, and there was no change in bladder weight between any of the treatment groups.

Intravesical instillations of gemcitabine into the bladder of animals have reported good tolerability. In studies of beagle dogs and pigs treated with repeated intravesical instillations of up to 350mg gemcitabine for four and six weeks respectively, no change in behaviour or overall well-being was reported during the treatment period (Cozzi et al., 1999, Witjes et al., 2003). The tolerability of intravesical gemcitabine found in the present study align with those found by Delto et al (2013), who reported no significant weight loss or signs of distress from mice treated with intravesical gemcitabine (25mg/mL).

### EFFECT OF GEMCITABINE ON VOIDING BEHAVIOURS

Voiding patterns of mice were measured pre- and post- intravesical gemcitabine or saline treatment. The voiding patterns and fluid intake of mice before treatment align with that

previously reported (Sugino et al., 2008, Boudes et al., 2011, Everaerts et al., 2010). The urodynamic parameters measured in mice treated with single and repeated instillations of gemcitabine significantly increased from pre- to post-treatment, indicative of change to a dysfunctional bladder phenotype. As increased frequency of urination is often reported in human patients undergoing intravesical gemcitabine treatment for bladder cancer, this result was not surprising (Dalbagni et al., 2006, Addeo et al., 2010, De Berardinis et al., 2004, Gontero et al., 2004). To the best of my knowledge, no studies have investigated the micturition behaviours in mice using similar voiding pattern analysis after intravesical chemotherapy. However, the development of cystitis-induced detrusor overactivity in mice is well documented, with reported increases in urination frequency as measured by voiding behaviours analysed with filter paper (Everaerts et al., 2010).

## **UROTHELIAL EFFECTS OF INTRAVESICAL GEMCITABINE**

Damage to the urothelial layer by intravesical gemcitabine was assessed by histological examination. While the luminal surface of the urothelium appeared intact, thickening of this epithelial layer was apparent in mouse bladders treated with single and repeated instillations of gemcitabine. Additionally, macroscopic signs of bladder inflammation including more prominent vasculature and areas of haemorrhage were noted in the gemcitabine treated mice bladders with comparison to the saline treated mice bladders (both single and repeated treatment groups). This has been similarly reported by Witjes et al (2003) in pigs after repeated instillations of intravesical gemcitabine. Furthermore, they noted mild signs of inflammation in the treated bladders, including subepithelial leukocyte infiltration and cellular and nuclear enlargement.

The toxicology of repeated intravesical instillations of mitomycin C in mice bladders has previously been assessed (Matsushima et al., 2011). Mitomycin C is the first-line chemotherapy option for NMIBC (Malmstrom et al., 2009, Hendricksen and Witjes, 2007), and is associated with significantly more side effects in patients with comparison to gemcitabine (72.2% compared to 38.8% respectively) (Addeo et al., 2010). Mice treated with intravesical mitomycin C at the clinical dose used in human patients (2mg/mL) succumbed during the experimental period due

to the severity of adverse effects associated with treatment (Matsushima et al., 2011). Pathological findings of the murine bladder showed acute inflammation, submucosal oedema and detachment of the urothelium from the underlying bladder wall.

It is well-reported that intravesical chemotherapy causes significant histological changes to the bladder wall. Installations of cytotoxic drugs into the bladder induce extensive denudation of the apical layer of the urothelium, followed by a period of rapid proliferation resulting in urothelial hyperplasia. The appearance of the remaining cells have been reported as atypical, including increase in cell size, multi-nucleated and vacuolated cells but with a normal nuclear/cytoplasmic ratio (Marušić et al., 2009, Choe et al., 1995, Drago et al., 1989, Castillo et al., 2012).

Urothelial thickening is characteristic of inflammatory conditions of the bladder (Wong-You-Cheong et al., 2006). Inflammation of the epithelial layer of the bladder has a direct effect on bladder function, including causing augmented release of urothelial mediators (ATP), increased sensitivity of the afferent nerves and altered muscle contraction (Birder et al., 2003, Grover et al., 2011). Mast cell activation by mediators released from damaged urothelium including substance P and nerve growth factor (NGF) induce a cascade release of inflammatory, vasoactive and nociceptive molecules, such as cytokines, nitric oxide, prostaglandins and vascular endothelial growth factor (Cao et al., 2005, Dubner and Ruda, 1992, Theoharides et al., 2001, Grover et al., 2011).

In addition to the histological changes induced by intravesical gemcitabine, the release of urothelial mediators ATP, acetylcholine and prostaglandin E<sub>2</sub> from the bladder was assessed. Release of mediators from mice bladders into the lumen and through the serosal wall was measurable and repeatable. When normalised to the volume of solution, the total release of each mediator was significantly greater from the serosal than the luminal side. This aligns with the findings of Ferguson et al (1997), who first reported the release of ATP from both the luminal and serosal surfaces in the rabbit bladder. Using an Ussing's chamber, they demonstrated that release of ATP is derived from the urothelium not detrusor, and bladder stretch induced an increase in release from the serosal but not luminal side. This was similarly observed by Dunning-Davies et al (2013), who also measured ATP release from the serosal side in response to stretch, mediated by the rise in intracellular [Ca<sup>2+</sup>]. As the apical layer of the

urothelium acts as a barrier to solutions and solutes and the targets of urothelial mediators lie in the sub-layers of the bladder, this mono-directional flow is not unexpected.

While there was no change in serosal release of ATP between treatment groups, the release of luminal ATP was significantly enhanced with repeated intravesical instillations of gemcitabine. It is well documented that ATP release from the urothelium plays an important role in the sensory response to bladder distension. Luminal applications of ATP have been found to induce bladder contraction in unanaesthetized rats, and acts as a signalling molecule of mechanosensation on the afferent nerves (Burnstock, 2007, Pandita and Andersson, 2002, Vlaskovska et al., 2001). Additionally, enhanced ATP has been measured from cultured urothelial cells from patients with interstitial cystitis, a disorder characterised by bladder inflammation, dysuria and increased frequency of urination (Sun et al., 2001). Similar to the findings of this study, enhanced ATP measured in the lumen of the bladder has been reported in rats with cyclophosphamide-induced cystitis, with no change in serosal release (Smith et al., 2005). Furthermore, increased luminal ATP release has been measured from an aged-mouse model with confirmed geriatric-bladder overactivity (Daly et al., 2014). The implications of these findings indicate that urothelial ATP release is enhanced, or that the ATP breakdown mechanisms are depressed after repeated instillations of gemcitabine. Enhanced urothelial ATP may act upon the underlying afferent nerves and muscle, particularly the nociceptive C-fibres (Kullmann et al., 2008), increasing sensory stimulation arising from the bladder. This may play a role in the side effects of intravesical gemcitabine for NMIBC reported by patients.

Acetylcholine release from both sides of the bladder was unchanged between treatment groups, while prostaglandin E<sub>2</sub> release through the luminal and serosal surfaces was significantly enhanced in bladders treated with repeated instillations of gemcitabine. As discussed in previous chapters, prostaglandin E<sub>2</sub> is released from the bladder with stretch, potentiated in the presence of the urothelium (Masunaga et al., 2006), and has been shown to be released from rat urothelial tissue in response to bladder distension (Tanaka et al., 2011). Furthermore, a study investigating prostaglandin E<sub>2</sub> release from the urothelium/lamina propria of the guinea pig bladder indicated that ATP can activate prostaglandin E<sub>2</sub> production mediated via COX-1 (Nile

and Gillespie, 2012). This corresponds with the enhanced ATP release observed from mouse bladders treated with repeated instillations of intravesical gemcitabine in this study.

Instillations of prostaglandin E<sub>2</sub> in the bladder has been shown to stimulate the nociceptive C-fibres but not A $\delta$ -fibres of the afferent nerves, and induces phasic contractions and increases muscle tone mediated via L-type calcium channels in rodent bladders (Aizawa et al., 2010, Kobayter et al., 2012). Additionally, elevated urinary levels of prostaglandin E<sub>2</sub> and tissue COX-2 expression have been measured in patients with overactive bladder symptoms and mouse models of inflamed bladders (Kim et al., 2006, Kim et al., 2005, Hu et al., 2003). This indicates that the enhanced release of prostaglandin E<sub>2</sub> measured in this study may play a role in the symptoms of overactive bladder seen within the mouse models treated with intravesical gemcitabine.

Damage to the urothelium and consequent increased permeability could be an argument for the increased luminal concentrations of urothelial mediators as measured in the present study. However, Smith et al. (2014) found enhanced luminal levels of both ATP and acetylcholine from porcine bladders treated with luminal DMSO. They concluded that the high levels reflected leakage of these mediators from the urothelium due to physical damage. As luminal acetylcholine concentrations were unchanged between control and gemcitabine treated mice, it is more likely that the enhanced ATP and prostaglandin E<sub>2</sub> concentrations are a result of increased release or depressed breakdown mechanisms.

Taken together, the altered voiding behaviours and histological appearance of mice treated with intravesical gemcitabine are indicative of inflammation of the urothelial lining of the bladder. Furthermore, enhanced release of ATP and prostaglandin E<sub>2</sub> are associated with inflammatory conditions of the bladder, and may play a role in altering the contractions of the detrusor and sensitizing afferent nerves.

## **BLADDER MUSCLE EFFECTS OF INTRAVESICAL GEMCITABINE**

It is well documented that the detrusor muscle exhibits spontaneous action potentials, underlying the myogenic phasic contractile activity of the bladder (Levin et al., 1986). Strips of

bladder tissue from patients with idiopathic detrusor instability (increased frequency and urgency) develop a greater spontaneous tone, but are less responsive to efferent nerve stimulation with EFS (Mills et al., 2000). Spontaneous contractile activity during bladder filling and accommodation has been shown in rodent isolated bladders, and is enhanced in models of cystitis-induced inflamed bladders and overactive detrusor after spinal cord transection (Yu and de Groat, 2008, McCarthy et al., 2009). Phasic activity was observed in all bladders tested in the current study, and there was no difference between the frequency and amplitude of contractions between mice treated with intravesical saline or gemcitabine. While intravesical gemcitabine treatment in mice induced an overactive bladder phenotype as measured by a change in voiding behaviour, it appears that the myogenic phasic contractions of the detrusor are not involved in this pathophysiology. This has been similarly reported by Daly et al (2014), who could find no changes in spontaneous frequency and amplitude of spontaneous contractions in mouse whole-bladder preparations from an aged-model of bladder overactivity.

Interestingly, mouse bladders treated with repeated instillations of intravesical gemcitabine displayed significantly increased compliance during filling, resulting in enhanced filling volume. This was an unexpected result, as patients suffering from inflamed bladders and detrusor overactivity are commonly characterised by reduced bladder compliance and bladder filling volumes to a fixed pressure (van de Merwe et al., 2008, Abrams et al., 2002). However, one of the sub-classifications of neurogenic bladder is characterised by over-distension due to bladder flaccidity, arising from detrusor muscle areflexia (Dorsher and McIntosh, 2012). Detrusor muscle areflexia can result from damage to the bladder efferent or afferent neural pathways, lumbosacral spinal cord damage or myogenic failure (Miyazato et al., 2013). From the results of the current study, it could be hypothesized that intravesical gemcitabine causes changes to the efferent and afferent nerves of the bladder, resulting in detrusor areflexia. Evidence for this will be explored further in this discussion.

To the best of my knowledge, this study is the first to investigate the functional effects of intravesical chemotherapy on the contractility and relaxation of the isolated whole bladder. Intravesical gemcitabine instillation caused no change between contractions mediated by the muscarinic or P2X purinergic receptors of the bladder. The addition of the NOS inhibitor L-NNA

had no effect on muscarinic contraction. Previous investigations of the contractility of cyclophosphamide-induced cystitis in mouse bladders suggested that the alteration of cholinergic detrusor responses is in part affected by enhanced nitric oxide production (Vesela et al., 2012). While this effect was not observed within this study, this may be due to the application of only one concentration (10 $\mu$ M) of carbachol. Furthermore, there was no difference in contraction to KCl between treatment groups, indicative that intravesical gemcitabine does not affect the intrinsic myogenic detrusor contraction. Finally, the relaxation of the whole bladder mediated by the  $\beta$ -adrenoceptors to isoprenaline was unchanged. These results are not entirely unexpected. As described previously, the penetrative effect of gemcitabine through the bladder wall is unknown, however the concentration of doxorubicin and mitomycin C are reduced by a 32- and 35-fold respectively (Wientjes et al., 1996, Wientjes et al., 1993). It could be assumed from the results of this study that the concentration of gemcitabine that reaches the detrusor muscle cells is not cytotoxic. Therefore, the enhanced voiding behaviours and increased compliance of mouse bladders observed after intravesical gemcitabine treatment are not myogenic in origin.

Physiological contraction of the bladder is initiated by parasympathetic nerves. Preliminary experiments found EFS of isolated mouse bladders produced repeatable, frequency dependent and TTX-sensitive contractions. Repeated, but not single, instillations of intravesical gemcitabine in mice significantly attenuated efferent nerve-mediated contraction to EFS.

The contribution of nitric oxide, muscarinic and P2X purinergic receptors to EFS mediated contraction was also investigated. In the control bladders, the addition of the nitric oxide synthase inhibitor L-NNA had no effect on contraction to any frequencies of stimulation. Antagonism of the muscarinic receptors with atropine significantly reduced contractions at low (1Hz) frequencies, with no effect at higher frequencies. Finally, the desensitization of P2X receptors reduced EFS mediated contractions by >70% at all frequencies. This is suggestive that the purinergic component makes up the majority of normal mouse bladder nerve-mediated contractions, while acetylcholine contributes at lower frequencies and NO has little to no effect.

Previous reports in the literature of EFS induced contraction in whole mouse bladders are inconsistent. The addition of the NOS inhibitor L-NOARG has been reported to have no effect

on bladder contractions induced by EFS (Fujiwara et al., 2000). However, Liu and Lin-Shiau (1997) found the addition of L-NOARG decreased neurogenic contraction in mouse detrusor strips, and this was reversed with the addition of L-arginine. Fabiyi and Brading (2006) measured almost identically sized responses of whole bladder and bladder strips to EFS, where the cholinergic and purinergic component of contraction made up approximately 50% respectively at all frequencies tested (1 – 35Hz). Similar to the present study, Lamarre et al (2014) reported an approximate 60% reduction in EFS mediated contraction with the addition of  $\alpha,\beta$ -mATP, while atropine affected only the low frequency-induced contractions. Furthermore, previous investigations (unreported) within our laboratory using whole mouse bladders align with the results of this study.

Repeated instillations of gemcitabine in mouse bladders significantly attenuated the contractile response to EFS. Aronsson et al. (2015) similarly measured depressed EFS response in cyclophosphamide treated rats, although also found that the reflex-evoked response is enlarged in these animals, possibly involving sensitization of sensory pathways. Interestingly, single and repeated instillations of intravesical gemcitabine in mouse bladders had no effect on the relative contribution of acetylcholine or ATP to EFS-mediated contraction with comparison to controls. However, the addition of L-NNA significantly enhanced the contraction to EFS at frequencies >5Hz. The role of nitric oxide in nerve-mediated contractions of the bladder is complex, as it has been reported to indirectly have both excitatory and inhibitory effects on the detrusor muscle (Andersson and Persson, 1994). As previously described in Chapter 1, nitric oxide has been implicated in a range of bladder pathophysiologies. Nitric oxide deficiency causes detrusor overactivity in rats through sensitization of muscarinic receptors and reducing  $\beta_3$  adrenoceptor mediated relaxation (Monica et al., 2008), while chronic irritation/inflammation of the rodent bladder causes increased neuronal and endothelial NOS expression (Kang et al., 2004, Vizzard et al., 1996). Furthermore, NOS inhibition has similarly been shown to increased EFS-mediated detrusor contractions in CPO-inflamed bladders of rats (Vesela et al., 2012). Models of chronic inflammation in rats show reduced contraction to EFS, and it has been suggested that this may be a result of enhanced NO acting prejunctionally on bladder nerves to inhibit acetylcholine release (Persson et al., 1993, Vesela et al., 2012).



It appears from the results of this study that nitric oxide plays a significant role in the reduction of efferent nerve mediated contraction after intravesical gemcitabine treatment. NO relaxation of the bladder is mediated through increased cGMP formation, with subsequent activation of cGMP-dependent protein kinase causing hyperpolarization of the detrusor muscle cell (James et al., 1993, Corcos et al., 2015). Furthermore, endogenously generated NO from CPO-inflamed rat bladders depresses the excitability of afferent nerves (Yu and de Groat, 2013). Aizawa et al (2011) found that NO has a direct inhibitory effect on the mechanosensitive A $\delta$ - and particularly the nociceptive C-fibres of the afferent nerves in anesthetized rats, which significantly enhanced bladder capacity during filling. The enhanced bladder capacity measured within the repeated gemcitabine treated mouse group in this study may be a result of this effect.

In summary, it appears that single and repeated instillation of intravesical gemcitabine to the mouse bladder does not directly affect the detrusor muscle ability in contraction and relaxation. However, it has an inhibitory effect on the efferent nerves and consequently may cause detrusor hyporeflexia, mediated most likely through enhanced nitric oxide release. In turn, this enhanced NO release may desensitizes mechanosensitive afferent nerves, which may result in enhanced bladder capacity.

## **CONCLUSIONS**

Single and repeated instillations of intravesical gemcitabine treatments in the mouse bladder resulted in altered voiding behaviours, namely an increase in small voids. The histological and macroscopic appearance of the urothelium showed signs of inflammation, releasing enhanced ATP and prostaglandin E<sub>2</sub>. Furthermore, efferent nerve mediated contractions were significantly depressed in gemcitabine treated bladders, likely due to enhanced NO release. The results of this study indicate that intravesical gemcitabine causes an overactive bladder phenotype, potentially mediated by enhanced urothelial release of ATP and prostaglandin E<sub>2</sub> acting on the C-afferents, and depressed efferent nerve mediated contractions. While this conclusion seems counterintuitive, there is significant evidence of the role of decreased motor innervation in detrusor instability, which will be discussed in the subsequent chapter.





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## **CHAPTER 6:**

### **GENERAL DISCUSSION**

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The aim of the present study was to investigate the mechanisms behind the reported side effects experienced by bladder cancer patients treated with intravesical gemcitabine. Consequently, this thesis has elucidated the effects of gemcitabine on the urothelium, nerves and detrusor muscle of the bladder. A variety of techniques and models were used during this investigation to measure changes in bladder function after gemcitabine treatment. Additionally, this thesis investigated cytotoxicity of gemcitabine with comparison to the current standard chemotherapy used for NMIBC, mitomycin C, and investigated the combination of gemcitabine and hyperthermia as an adjunct treatment. This final discussion will review the experimental models used within this thesis, gemcitabine use in patients, and specifically examine the changes within each tissue type after intravesical gemcitabine treatment and the consequent interactions and contribution to overall bladder functioning.

## **COMPARISON OF EXPERIMENTAL MODELS**

Multiple experimental models were used for the present studies, each with advantages and limitations. *In vitro* urothelial cell culture models have been used previously to assess cell-specific responses to various stimuli (McDermott et al., 2012, Kang et al., 2013a, Kang et al., 2015, Mansfield and Hughes, 2014a, Mansfield and Hughes, 2014b). Similarly, this model was used in the present study to examine the cytotoxicity of gemcitabine on malignant and non-malignant urothelial cells derived from human tissue. The functional effect of gemcitabine treatment on the non-malignant UROtsa urothelial cell line was also examined, which exhibit structural and functional properties analogous with *in vivo* models (Rossi et al., 2001). Furthermore, cell culturing provides a cost effective, controlled environment in which experiments investigating both the effects immediately after treatment and subsequent recovery periods are easily performed. However, the barrier integrity of the bladder organ, the cell-to-cell interactions and systemic input is not well represented in this model, which must be kept in mind when drawing conclusions that relate to the clinical setting.

As previously discussed in Chapter 4, porcine tissue is comparable to human in both structure and function. This tissue was used in the present study to examine the effect of gemcitabine on a full-thickness bladder wall model in which all cell types of the bladder (urothelial/lamina propria, myofibroblasts, nerves and smooth muscle) and a complete barrier (GAG layer and

uroplakins) were present. Furthermore, the drug administration in this model mimicked the conditions of intravesical treatment, where gemcitabine (40mg/mL) was applied only to the urothelial side for 60 minutes at 37°C. However, like the cell culture method, the systemic influences associated with organ blood flow are not represented in this model. Thus, penetration of gemcitabine through the bladder wall may be greater than in patients in this model and the immune responses will also be absent. Furthermore, tissue viability in this model is limited, which prevents the examination of recovery and multiple treatments.

Finally, *in vivo* effects of gemcitabine treatment were examined in mice. This model represents true intravesical treatments to an intact bladder system with the associated systemic blood flow and inflammatory/immune response. Furthermore, the treatment schedule in mice mirrored that of the clinical setting, where repeated treatments were given after intermittent recovery periods. Voiding pattern analysis complemented *ex vivo* isolated bladder studies, which elucidated both the physiological and functional effects of true intravesical treatment with gemcitabine. However, there are significant differences between the physiology of mouse and human bladder tissue as discussed in Chapter 5, which must be considered when interpreting the results of these studies.

## **GEMCITABINE USE IN PATIENTS**

At presentation, up to 80% of all bladder cancers are diagnosed as superficial or NMIBC. Intravesical gemcitabine is only a relatively new treatment for NMIBC, with proven cytotoxicity against tumours and excellent tolerability in Phase I, II and III trials in human patients (Jones et al., 2012, Shelley et al., 2011a, Witjes et al., 2004, Gontero et al., 2004, Dalbagni et al., 2006, De Berardinis et al., 2004, Bartoletti et al., 2005, Addeo et al., 2010). However, the current first-line and most commonly used intravesical chemotherapeutic agent is mitomycin C, which is associated with a significantly greater rate of side effects in patients than gemcitabine, including dysuria, increased frequency and urgency and chemical cystitis (Griffiths, 2012, Addeo et al., 2010).

This thesis compared the cytotoxicity of gemcitabine and mitomycin C on cultured normal urothelial cells in addition to bladder cancer cell lines. It was discovered that while both drugs

had similar potency on the cancer cells, gemcitabine was significantly less cytotoxic than mitomycin C on the normal urothelial cells. As damage to the urothelial cells by chemotherapies is hypothesized to be one of the mechanisms underlying the reported side effects of treatment, it would be a judicious change for gemcitabine to be given to patients as the first line chemotherapy agent instead of mitomycin C.

An adjunct regime investigated for bladder cancer treatment is localised hyperthermia, used in combination with chemotherapy. The efficacy and toxicity profile of combined gemcitabine and hyperthermia treatment in bladder cancer patients has not been reported in the literature. This thesis provides a basic investigation of the cytotoxic and consequent tissue changes with combined hyperthermia and gemcitabine. Firstly, there was no synergistic cytotoxic effect of the combined therapy on bladder cancer cell lines, while normal urothelial cells appeared to be more sensitive to this regime. Secondly, the tissue effects of luminal gemcitabine treated at hyperthermic conditions further depressed myogenic and efferent nerve-mediated responses compared to gemcitabine alone. Furthermore, the sloughing of the apical urothelial cells was more pronounced in the combined treatment. It appears from these preliminary observations that there is limited benefit in a combined hyperthermia and gemcitabine treatment regime for bladder cancer patients. However, the cytotoxic effects of hyperthermia for cancer treatment are also mediated by vascular damage and the induction of an immune response, and these parameters were not tested within this study (Rampersaud et al., 2010). Further investigation *in vivo* is needed to elucidate whether combined gemcitabine and hyperthermia treatment has a therapeutic benefit in the treatment of bladder cancer in humans.

## **SOURCES OF BLADDER DAMAGE AND INFLAMMATION BY GEMCITABINE**

Inflammation of the bladder is the first response to urothelial damage or exposure of the bladder to noxious stimuli. It is suggested that the cascade of mediators stimulated by chronic inflammation may sensitize bladder afferent nerves and is thought to contribute to bladder overactivity and pain (Dang et al., 2008, Hayashi et al., 2009). It has been shown in animal models of chronic inflammation with interstitial cystitis induced by cyclophosphamide that



expression of nitric oxide synthase, neuropeptides, growth proteins, COX-2 and prostaglandins in bladder afferent neurons are up-regulated (Vizzard et al., 1996, Vizzard and Boyle, 1999, Vizzard, 2001, Yoshimura et al., 2014). In particular, hyperactivity and enhanced mechanosensation of the C-fibre afferents in response to distensions of the inflamed bladder have been suggested to be causative of the pain and overactivity experienced by patients with interstitial cystitis (Yoshimura et al., 2014, Yoshimura et al., 2002). The side effects reported by patients undergoing intravesical gemcitabine are similar to that of interstitial cystitis, including increased frequency and urgency of urination, dysuria and haematuria. Furthermore, it has been previously reported that doxorubicin and mitomycin C induce enhanced and prolonged release of inflammatory mediators including nitric oxide, prostaglandin E<sub>2</sub> and cytokines IL-8 and IL-1 $\beta$ , suggested to be responsible for the urological side effects reported by patients undergoing intravesical treatment with these agents (Kang et al., 2013a, Kang et al., 2015). The results presented in this thesis, supported by previous findings, indicate the induction of bladder inflammation by gemcitabine as a possible mechanism of bladder dysfunction. Consequently, the following discussion will address the tissue specific changes of the bladder after gemcitabine treatment, and the possible role of inflammation in the etiology of reported symptoms of bladder overactivity and pain.

## **EFFECT OF GEMCITABINE ON DETRUSOR MUSCLE**

While luminal gemcitabine depressed myogenic activity of the detrusor muscle immediately after treatment, this effect appeared to recover after a 24 hour period as measured *ex vivo* in mice. Furthermore, the results presented in the present studies did not show any effect of gemcitabine on the spontaneous activity of the bladder. Previous studies have shown alterations of the phasic contractions in rodents with detrusor overactivity and inflamed bladders (McCarthy et al., 2009), however this may be a long term effect of chronic irritation or inflammation of the bladder.

These results suggest that either luminal gemcitabine does not penetrate the bladder wall through to the muscle layer, or that it is not directly cytotoxic to the muscle cells. To the best of my knowledge, no studies have investigated the direct effect of gemcitabine on smooth muscle tissue. An *in vivo* orthotopic pancreatic cancer rat model treated with repeated doses of

gemcitabine (100mg/kg) over 120 days found expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) to be significantly reduced in myofibroblast-like cells (Yamao et al., 2013). While tenuous, this evidence may indicate that long-term treatment with gemcitabine may influence muscle contractility, either directly through depressed actin expression in muscle or via reduced myofibroblast activity.

## **EFFECT ON UROTHELIAL LAYER**

The principle function of the urothelium is to act as distensible barrier to water, solutes and ions, protecting the underlying nerves and detrusor muscle of the bladder. This barrier function is regulated by a variety of factors, including hormones, neurotransmitters, proteases, cytokines and exposure to noxious chemicals (Lewis et al., 1995, Lavelle et al., 2002). The permeability barrier of the urothelium is compromised in various urinary bladder disorders including interstitial cystitis (painful bladder syndrome) and overactive bladder (Parsons, 2007, Lavelle et al., 2000, Parsons et al., 1991, Teichman and Moldwin, 2007, Parsons, 2011). This has been shown in patients with interstitial cystitis, where 25% of a solution of concentrated urea was absorbed through the bladder wall, in comparison to only 4.3% in normal healthy subjects (Parsons et al., 1991). It is thought that the passing of noxious substances through the bladder wall, acting upon the underlying muscle and nerves results in the symptoms of overactivity and pain.

Inflammation of the urothelial layer has also been reported to occur with these disorders with disrupted permeability (Grover et al., 2011, Steers, 2002), however it is currently unclear whether urothelial damage directly causes inflammation, or if it develops as a secondary effect. In a rat-model of urothelial injury using intravesical instillations of protamine sulphate (PS), bladder inflammation occurred in PS treated mice, but not nephrectomised PS treated mice (Soler et al., 2008). This indicates that disruption of the permeability layer does not directly cause inflammation, but instead allows transport of noxious substances through the bladder wall which in turn stimulates the inflammatory process. Furthermore, Lavelle et al (2002) measured the transepithelial resistance following PS-induced injury in rats, observing significant increases in permeability of the bladder wall up to 2 days post-injury, with gradual declines to control levels 3-5 days after PS exposure. Interestingly, the severity of inflammation measured by light

microscopy over 10 days post-PS instillation correlated with the permeability pattern of the bladder (Lavelle et al., 2002).

Conclusive evidence of the cytotoxic and damaging effect of gemcitabine on urothelial cells was obtained in all three results chapters of this thesis. Specifically, urothelial sloughing of the umbrella cells and disruption to the apical integrity of the urothelium was observed immediately after luminal application of gemcitabine on porcine bladder tissue. However, a 24-hour recovery period after intravesical gemcitabine treatment in mice showed thickening of the urothelium with comparison to control bladders, indicative of induction of a cellular recovery/proliferation process.

The cells of the normal urothelium have a low cellular turnover rate and display very little mitotic activity (Martin, 1972). However, this turnover is rapidly increased after exposure to cytotoxic agents, bacteria and bladder damage (Simeonova et al., 2000, Uchida et al., 1989, Romih et al., 1998). The molecular mechanisms that stimulate tissue repair are not entirely understood, however growth factors, namely epidermal growth factor (EGF) and the corresponding epidermal growth factor receptor (EGFR), have been confirmed to be involved in urothelial regeneration (Daher et al., 2003). EGFR expression is limited to the basal cells of the urothelium, with only scattered representations in the intermediate cell layer (Messing, 1990). The presence of the protective GAG-layer and lack of EGFR on the umbrella cell layer prevents EGF present within urine from inducing urothelial proliferation (Cheng et al., 2002). However, exposure of the underlying urothelial layers to EGF after bladder injury stimulates the regenerative process, leading to cellular proliferation, hyperplasia and eventual differentiation (Romih et al., 2001, Varley and Southgate, 2008). This supports the findings of this thesis.

## UROTHELIAL RELEASE OF INFLAMMATORY MEDIATORS

The release of ATP and prostaglandin E<sub>2</sub>, but not acetylcholine from urothelial cells is altered after gemcitabine treatment. In human urothelial cells and mouse bladder, the release of ATP from the urothelium was enhanced after luminal treatment of gemcitabine. It is well reported that ATP is released from the bladder in response to inflammation (Kumar et al., 2007, Sun et al.,

2001, Smith et al., 2005). However, there is some debate as to the mechanism of this enhanced ATP release. A recent study by Mansfield and Hughes (2014a) found that the release of inflammatory mediators bradykinin, histamine and serotonin were unlikely to be responsible for increased ATP release in human urothelial cells. As ATP is released from urothelial cells with mechanical stimulation via exocytosis mediated by the soluble N-ethylmaleimide sensitive factor receptor (SNARE) complex, it has been suggested that these SNARE mechanisms may be up regulated after bladder inflammation (Khera et al., 2004, van der Wijk et al., 2003). Furthermore, botulinum toxin A acting via the inhibition of SNARE-dependent exocytotic processes in urothelial cells has been shown as a successful treatment for the symptoms of overactive bladder and acts via a reduction in stretch-induced release of urothelial ATP (Khera et al., 2004, Schmid et al., 2006, Apostolidis et al., 2006, Hanna-Mitchell et al., 2015).

Enhanced release of urothelial ATP has numerous targets and consequent alterations in sensory and mechanical stimulation within the bladder. It has been shown that urothelial ATP acts upon the purinergic receptors on the A $\delta$ - and C-fibres of the mechanosensitive and nociceptive afferent nerves (Burnstock, 2009). Furthermore, urothelial ATP may influence the underlying myofibroblasts and detrusor muscle, and can act in an autocrine manner on the urothelial cells itself (Wang et al., 2005, Birder and Andersson, 2013). Stimulation of the urothelial P2Y receptors by enhanced ATP release after UROtsa cell infection with UPEC (*Escherichia coli* bacteria) has been shown to cause increased release of the inflammatory cytokine interleukin-8 (Save and Persson, 2010). As found in this study, cytokines including IL-1 $\beta$ , IL-6 and IL-8 have been found to be released from urothelial cells in response to both infection and damage induced by cytotoxic drugs (Kang et al., 2013a, Funfstuck et al., 2001). These pro-inflammatory mediators have a multitude of targets within the bladder, including the process of pathological pain, recruitment of inflammatory cells (T-cells, mast cells and neutrophils), cellular proliferation and enhanced release of prostaglandin E<sub>2</sub> and substance P (Zhang and An, 2007, Schweizer et al., 1988).

This inflammatory response has also been shown to induce production of reactive oxygen species (Mulvey et al., 2000). Upregulated ROS production has recently been linked to afferent nerve sensitivity. Ruan et al. (2014) recently demonstrated the sensitizing effect of ROS on

vagal lung afferent C-fibres, likely mediated through the activation of the TRPV1, TRPA1 and P2X receptors. They found that the sensitized afferents resulted in an increased electrical excitability and consequent exaggerated reflex responses in rat airways (Ruan et al., 2014). While this effect was measured in the lung, this may be a further mechanism of afferent sensitivity in the bladder. In the present thesis, gemcitabine induced ROS production in urothelial cells, which may therefore play a role in sensitizing afferent nerves, causing symptoms of bladder overactivity and pain reported by patients after intravesical treatment with this agent.

Masuda et al. (2008) reported that intravesical instillation of hydrogen peroxide into the bladders of anaesthetized rats induced the micturition reflex, and they suggested this effect was mediated through the afferent fibres by either direct activation of the C-fibre neurons or indirectly by stimulating the release of mediators. Interestingly, they noted that prostaglandin E<sub>2</sub> and F<sub>1α</sub> content was enhanced in hydrogen peroxide stimulated bladders, and the addition of nonselective COX inhibitors indomethacin and ketoprofen suppressed the excitatory effects of hydrogen peroxide (Masuda et al., 2008). Taken together, this indicates that the excitatory afferent response due to ROS may be mediated by stimulation of the COX pathway. Additionally, enhanced ROS formation in lung tissue is known to increase the release of arachidonate metabolites (Matyas et al., 2002), although this effect in bladder tissue has not been reported. Interestingly, Eblin et al (2007) also noted that the transformation of normal human urothelial cells to a proliferative phenotype was correlated with enhanced ROS production and COX-2 expression after acid-induced damage.

Urothelial COX-2 expression is up-regulated in conditions of bladder obstruction, interstitial cystitis, inflammation and overactive bladder (Hu et al., 2003, Park et al., 1999, Jang et al., 2006). Correspondingly, prostaglandin E<sub>2</sub> release is enhanced in response to urothelial damage and urinary concentrations are increased in patients with interstitial cystitis (Wheeler et al., 2002, Shioyama et al., 2008). In the present studies, the release of prostaglandin E<sub>2</sub> from the urothelium of pig and mouse bladders was found to be enhanced after luminal gemcitabine treatment.

Like ATP, prostaglandin E<sub>2</sub> is released by the urothelium and has multiple targets within the bladder (Poggesi et al., 1980). As discussed previously, prostaglandin E<sub>2</sub> has a role in directly increasing bladder tone and stimulating nociceptive C-fibers on the afferent nerves. However, Schroder et al (2004) reported that the role of EP1 receptors (prostaglandin E<sub>2</sub> receptor 1) do not appear to be essential for normal micturition, but do play a role in the development of detrusor overactivity. It has also been suggested that prostaglandin E<sub>2</sub> given intravesically to the rat bladder may indirectly facilitate detrusor contraction by the release of tachykinins from bladder nerves, initiating a micturition reflex through the stimulation of the NK-1 and NK-2 receptors (Ishizuka et al., 1995, Andersson, 2002). Furthermore, prostaglandin E<sub>2</sub> plays a complex role in the inflammatory response of tissues. It is generally recognised as a mediator of active inflammation, attracting neutrophils, macrophages and mast cells and inducing production of inflammatory cytokines including IL-6 and IL-8 (Yu and Chadee, 1998, Kozawa et al., 1998, Nakayama et al., 2006, Weller et al., 2007).

Gemcitabine treatment on the bladder and urothelial cells enhanced urothelial release of ATP, prostaglandin E<sub>2</sub>, inflammatory cytokines IL-1  $\beta$ , IL-6 and IL-8 and stimulated ROS production. Taken together, these observations suggest that the physical damage to the apical urothelial cells and consequent release of mediators by gemcitabine treatment may sensitize underlying afferent nerves, inducing an inflammatory response that may sensitize the afferent nerves and alter the normal functioning of the efferent bladder nerves. The following sections will further elucidate the reasoning for this hypothesis.

## **EFFECT OF GEMCITABINE ON AFFERENT NERVES**

While this study did not investigate the direct effect of intravesical gemcitabine on the afferent nerves of the bladder, there is some evidence that indicates that sensitization of these nerves may play a role in the symptoms of pain and overactivity of the bladder reported by patients.

As covered previously, the two main types of afferent nerves within the bladder are the myelinated A $\delta$ -fibres and the more common C-fibres, which sense mechanical, thermal and chemical stimuli and relay information regarding storage and micturition to the central nervous system (Kanai and Andersson, 2010). These distension sensitive bladder afferents are further

distinguished by their low (<15mmHg) and high (>15mmHg) intravesical mechanical thresholds of stimulation, which are conducted by the A $\delta$ - and C-fibre ranges respectively (Shea et al., 2000). While A $\delta$ -fibres are located primarily in the detrusor smooth muscle layer and respond to detrusor stretch during bladder filling, C-fibres are found within the detrusor and the sub-urothelium/lamina propria regions, and are postulated to play a role in volume, chemical and thermal sensations (Wakabayashi et al., 1993, Xu and Gebhart, 2008).

Afferent nerve sensitisation (lowered thresholds) has been postulated to play a role in the symptoms of painful and overactive bladder, particularly the C-fibre afferents. In patients with detrusor overactivity caused by bladder outlet obstruction but not in normal subjects, selective C-fibre stimulation by ice-water induced the micturition reflex, suggesting up-regulation of these afferents (Hirayama et al., 2003). Intravesical instillation of the C-fibre specific neurotoxins resiniferatoxin (RTX) and capsaicin have also been used clinically for patients of bladder overactivity, delaying involuntary detrusor contractions during filling cystometry and reducing the sub-urothelial afferent nerve densities respectively (Silva et al., 2002, Dasgupta et al., 2000). Furthermore, enhanced release of urothelial mediators including ATP and prostaglandin E<sub>2</sub> are well documented to play a role in the sensitization of afferent nerves, particularly the C-fibre afferents (Burnstock, 2009, Lin et al., 2006).

## NEUROGENIC INFLAMMATION

The peripheral terminals of sensory neurons within tissues release a number of bioactive mediators in response to local depolarization, axonal or dorsal root reflexes. In turn, these substances act on target cells in the periphery including mast cells, immune cells and vascular smooth muscle inducing inflammation characterised by redness, swelling and neuronal hypersensitivity. Collectively, these symptoms resulting from the release of substances from sensory nerve terminal are termed neurogenic inflammation (Richardson and Vasko, 2002). In the bladder, the inflammatory neuropeptides released from sensory nerve terminals in response to noxious stimuli include substance P, calcitonin gene-related peptide (CGRP) and neurokinin A (Geppetti et al., 2008). These neuropeptides acting via their respective receptors induce tissue oedema and neutrophil accumulation, are implicated in the production of reactive oxygen

species and promote bladder contraction through stimulation of receptors present on the bladder nerves.

The ability of capsaicin and other irritants to produce sensations of pain is mediated by the TRP vanilloid 1 channel (TRPV1), which is present in the primary afferents and urothelial cells of the bladder (Avelino et al., 2002, Birder et al., 2001). Furthermore, increased expression of TRPV1 channels and nerve growth factor (NGF) has recently been shown *in vivo* in conditions of bladder inflammation and hyperactivity (Liu et al., 2009, Dornelles et al., 2014). NGF is produced by the urothelium and detrusor muscle, stimulating several intracellular signalling cascades that regulate neuronal responses with roles in pain-signalling systems (Pezet and McMahon, 2006, Steers and Tuttle, 2006). Using *in vitro* approaches, Stein et al (2006) and Coelho (2015) observed that NGF regulated the expression and function of TRPV1 channels on neuronal and urothelial cells respectively. This enhanced receptor expression was correlated with increased sensitivity to noxious stimuli, which supports the role of TRPV1 and NGF in the induction of neurogenic inflammation.

Commonly known as the wasabi receptor, TRPA1 (transient receptor potential cation channel, subfamily A, member 1) is co-expressed with TRPV1 in sensory nerves of the bladder (Story et al., 2003). These receptors are activated by cold stimuli and the products of tissue injury including oxidative stress and environmental irritants, and promote neural pain, neuropeptide release and neuronal inflammation (Trevisani et al., 2007, Bautista et al., 2006).

Mast cells have also been implicated in neurogenic inflammation. Chemical, immune and mechanical irritation of the bladder, in addition to conditions characterised by overactive and painful bladders are associated with morphological changes in sensory and motor neurons and the number and distribution of mast cells (Dupont et al., 2001, Sant and Theoharides, 1994). These cells are induced to sites of inflammation by numerous trigger mediators, including cytokines, free radicals, NGF, substance P, acetylcholine, noxious chemicals and neuropeptides (Theoharides et al., 2001). Furthermore, substance P released by the afferent nerves in the bladder stimulates the degranulation of mast cells (Ercan et al., 2006), which release cytokines/chemokines, growth factors, peptides and proteoglycans (Theoharides et al., 2001). These mediators in turn attract leukocytes to the site of inflammation, through chemokine



receptors present on leukocytes sensing increasing chemotactic concentration gradients and facilitating cellular infiltration towards them (Rutkowski and DeLeo, 2002).

Subpubic pain, dysuria and increased frequency and urgency are common complaints from bladder cancer patients undergoing intravesical chemotherapy. There is limited evidence as to an explanation for these symptoms; however it appears likely that neurogenic inflammation is in part responsible. With repeated instillations of intravesical gemcitabine into pig bladders, signs of the inflammatory response were evident (Witjes et al., 2003). Specifically, leukocyte infiltration to the epithelial layer was noted, which is associated with cascades of inflammatory mediators, including cytokines, histamines, kinins, nitric oxide and proteases (Grover et al., 2011). Furthermore, *in vivo* investigations into the inflammatory response after intravesical mitomycin C following transurethral resection has been performed in mice. Chronic inflammation was caused by repeated instillations of mitomycin C, associated with enhanced lymphocyte and eosinophil migration to the bladder, oedema and fibrosis. It has previously been shown that leukocytic adhesion is mediated by NK1 receptors after stimulation with the inflammatory neuropeptides substance P, where release is enhanced during periods of bladder neurogenic inflammation (Baluk et al., 1995).

## **EFFECT OF GEMCITABINE ON EFFERENT NERVES**

Intravesical instillation of gemcitabine depressed the efferent nerve-mediated contractions induced by EFS both immediately and after 24-hours recovery on porcine and mouse bladder respectively. The synaptic release of the neurotransmitters acetylcholine and ATP are the main drivers of detrusor contraction; however numerous substances including monoamines, purines, amino acids, peptides and NO have also been implicated as NANC neurotransmitters and play a role in bladder contraction (Zhou and Ling, 1999). Parasympathetic nerves terminating in the detrusor muscle of the human bladder stain positively for acetylcholinesterase and for vesicular acetylcholine transferase, indicating that the release of acetylcholine is vesicular (Ek et al., 1977, Dixon et al., 1983). ATP is co-released with acetylcholine by efferent nerves of the bladder similarly by exocytosis, although there is some debate as to the exact release mechanism (Burnstock et al., 1978, Bodin and Burnstock, 2001).

Depressed nerve-mediated contraction of the bladder could be a result of numerous alterations in efferent activity. These include direct damage to the efferent nerves, impairment of or alterations to neurotransmitter release or depressed myogenic mechanisms. After immediate luminal treatment of gemcitabine on porcine bladder, it appeared that the depressed efferent-nerve mediated contractions were due to direct muscle damage, due to loss of intrinsic contractility with KCl, muscarinic and purinergic stimulation. However, in mice bladders 24-hours post repeated intravesical gemcitabine (ie. recovery); neurogenic responses to EFS were significantly depressed without any change in muscle contractility. The addition of a nitric oxide synthase inhibitor L-NNA significantly enhanced contraction amplitude with no changes to the contribution of acetylcholine or ATP, indicative of enhanced neurally released NO after gemcitabine treatment. It would be my hypothesis that the depressed contraction of gemcitabine pre-treated porcine bladder to EFS is mediated in part by enhanced NO release, however this effect was not examined.

In the guinea-pig and human bladder, NOS-immunoreactive nerve fibres and intrinsic neurons are distributed throughout the bladder body (Smet et al., 1996). NO is released from parasympathetic nerves with acetylcholine, and exerts an inhibitory effect on contractions (Dokita et al., 1991). In the urinary bladder of mice with cyclophosphamide-induced cystitis, but not control mice, a NO-dependent reduction in muscarinic contraction has been reported in addition to a decrease in EFS-induced contractions (Giglio et al., 2005, Giglio et al., 2007). Vesela et al. (2012) tentatively suggested that the reduction in contractility of inflamed mouse bladders to EFS is the result of an enhanced production of NO and consequent relaxation. Furthermore, NO is thought to play a role in inflammation progression, as elevated levels of NO have been reported in inflammatory diseases including bacterial and interstitial cystitis and after BCG treatment for bladder cancer (Ehren et al., 1999a, Logadottir et al., 2004, Lundberg et al., 1996). Additionally, urothelial cells treated with mitomycin C released more nitric oxide than control cells persistently over a prolonged period (Kang et al., 2015). It would be an interesting avenue of future research to investigate the effect of gemcitabine on nitric oxide release from the bladder, and its consequent effects on the detrusor muscle.

Although somewhat counter-intuitive, the physiological implications of depressed efferent nerve induced contractions of the bladder after intravesical gemcitabine may contribute to the symptoms of overactivity reported by patients. Detrusor underactivity is a common lower urinary tract dysfunction that is poorly understood, but is characterised by impaired bladder emptying that is distinct from bladder outlet obstruction (Abrams et al., 2002). Furthermore, the impaired detrusor contractility may arise from either a reduction in contractile strength and/or duration resulting in prolonged or incomplete emptying, which in turn can lead to increased voiding habits (Osman et al., 2014). Some of the causes of detrusor underactivity include neurogenic and myogenic origins, aging or medication side effects (Miyazato et al., 2013). Disruption to, or decline in, the efferent nerve activity can result in detrusor underactivity and is typically seen in diseases involving neuronal injury, in aged human bladders and in patients with bladder outlet obstruction (Gilpin et al., 1986, Gosling et al., 1986).

## **FUTURE INVESTIGATIONS**

The culmination of this thesis has generated a number of questions and avenues for future investigation. Gemcitabine cytotoxicity on urothelial cells is associated with enhanced reactive oxygen species production, and induces augmented release of mediators ATP and prostaglandin E<sub>2</sub> and cytokines. Taken together, these findings indicate that gemcitabine plays a role in initiating an inflammatory response in the bladder. As previously discussed, afferent nerve sensitization is associated with inflammatory conditions of the bladder and the symptoms of overactivity and pain. Consequently, it would be an interesting avenue of research to investigate the effect of intravesical gemcitabine, in addition to other commonly used chemotherapeutics for bladder cancer, on total afferent nerve activity and more specifically individual nerve fibres.

From a clinical perspective, this thesis provides evidence that the symptoms of bladder overactivity and pain experienced by patients undergoing intravesical therapy with gemcitabine are most likely linked to the induction of inflammation within the bladder. It would appear from this that anticholinergic medications that are currently used as the first line treatment for bladder overactivity would thus be ineffective in relieving patient symptoms. Furthermore, it has recently been shown that patients undergoing intravesical BCG with oral oxybutynin (anticholinergic medication) had a greater increase in urinary frequency and pain than the placebo group (Johnson et al., 2013). Thus, there may be therapeutic benefits of anti-inflammatory medication over the standard anticholinergics to address the symptoms of bladder overactivity reported by patients undergoing intravesical gemcitabine, warranting further investigation.

The penetrative ability of gemcitabine through the urothelium is currently unknown. Accordingly, this would be beneficial for future studies elucidating the direct effects of this chemotherapeutic on the bladder efferent nerves and detrusor muscle. This thesis focused on the effects immediately post-gemcitabine treatment and after short-term recovery periods. Further research into the effects of repeated intravesical gemcitabine treatment and long-term recovery in animal models would further elucidate the sustained tissue damage and consequent cellular changes.

## CONCLUDING REMARKS

The results presented here indicate that intravesical instillations of gemcitabine cause symptoms of bladder overactivity, as assessed by increased voiding behaviours in a mouse model. The cytotoxicity of gemcitabine is significantly more potent on malignant human urothelial cells with comparison to non-malignant urothelial cells. Furthermore, it is likely that enhanced release of urothelial mediators and inflammatory cytokines, and depressed efferent nerve activity after gemcitabine treatment play a role in the pathogenesis of bladder overactivity and pain reported by patients, through sensitization of bladder afferent nerves and reduced detrusor muscle contractility. It also appears from the results of this thesis that gemcitabine may be a superior treatment option for patients with non-muscle invasive bladder cancer than the current first-line agent mitomycin C, due to its enhanced cytotoxicity on malignant cells and improved toxicity profile.

In conclusion, gemcitabine is less cytotoxic to normal urothelial cells than malignant cells, but still has a substantial impact on normal bladder function which can explain the symptoms of bladder overactivity and pain observed in patients.





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